JOURNAL OF AGRICULTURAL RESEARCH

VOL. 57 WASHINGTON, D. C., DECEMBER 15, 1938 No. 12

RESPIRATION AND LACTIC ACID PRODUCTION BY A FUNGUS OF THE GENUS RHIZOPUS[1]

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INTRODUCTION

Lactic acid formed in living systems is usually considered the characteristic product of the anaerobic or fermentative phase of metabolism. Among the micro-organisms, this acid was formerly believed to be associated with the activities of certain specific bacteria, found among several groups of anaerobic organisms, comprising both rod-shaped and spherical forms. Recently, however, it has been definitely established that this acid is also produced during the growth of certain fungi belonging largely to the Mucorales. Although these fungi have long been known as capable of growing both under aerobic and anaerobic conditions, the final product of their anaerobic metabolism has usually been found to be an alcohol. On the other hand, lactic acid is produced by some of these fungi in an aerobic environment, with the organisms forming a pellicle on the surface of the medium; even when the cultures are thoroughly aerated, the acid still is formed. One may, therefore, be justified in asking whether the lactic acid thus produced by the fungi is a result of the aerobic oxidation of the carbohydrate or whether it is due to an anaerobic phase of their metabolism. In other words, can lactic acid be a product of normal respiration, that is, of the oxidative activities of the fungi, or do the fungi possess, like animals and higher plants, a fermentative or glycolytic stage in their metabolism.

The following experiments deal with the nutrition of a fungus belonging to the genus *Rhizopus*,[2] its ability to produce lactic acid from different carbohydrates, the mechanism of production of this acid, and the role of this process in the metabolism of the fungus.

METHODS

It has previously been shown (*30*)[3] that the species of *Rhizopus* used in the work reported in this paper grows readily on simple inorganic media containing glucose or starch as sources of energy and carbon for cell synthesis. When $CaCO_3$ was present in the medium, abundant lactic acid was produced and accumulated in the medium. In the absence of a neutralizing agent, lactic acid was formed but it accumulated only until the limiting reaction was reached, namely, about pH 4.0; however, growth of the organism continued even after this reaction had been attained.

In order to measure the evolution of CO_2 during the growth of the organism, KOH or NaOH solutions were used in some experiments

[1] Received for publication Mar. 21, 1938. Issued February 1939. Journal series paper of the New Jersey Agricultural Experiment Station, Department of Soil Chemistry and Microbiology.

[2] Of the group *R. arrhizus* or *R. nodosus*.

[3] Italic numbers in parentheses refer to Literature Cited, p. 898.

to neutralize the acid formed. The addition of the alkali was begun after growth of the fungus was well established. Because of the detrimental effect of even a slight excess of the alkali on germination of the fungus spores, care was exercised not to render the medium too alkaline. An excess of alkali can be avoided by the addition of an indicator to the medium; bromcresol purple, at the rate of 1 cc of a 0.5-percent solution per 100 or 200 cc of medium, was found to be suitable for this purpose. The sterile alkali solution, 0.25 to 1.0 N, was added directly to the culture until a change in color at about pH 6.0 had been attained. An arrangement for the growth of the organism, for the neutralization of the acid produced, and for measurement of the CO_2 liberated in the process of growth is shown in figure 1. The CO_2 was absorbed in a standard $Ba(OH)_2$ solution and the excess barium titrated with standard oxalic acid solution.

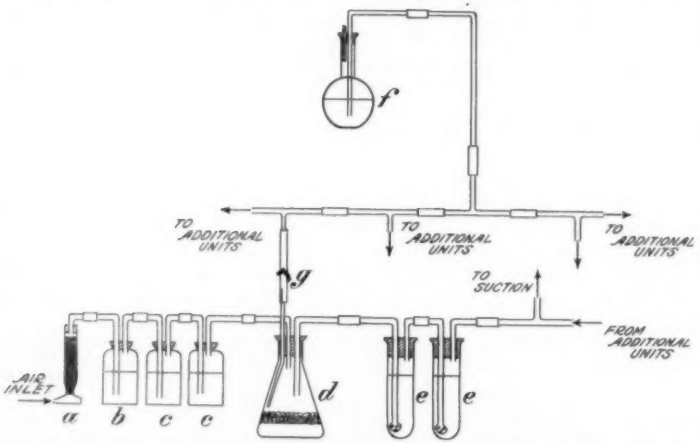

FIGURE 1.—Apparatus for neutralization of acid formed and for measurement of CO_2 evolved in the growth of fungi: Sterile 0.25 N KOH is introduced from reservoir (*f*) into culture flask (*d*) by means of stopcock (*g*). The flask is shaken gently in rotary fashion during the addition of alkali until the desired pH value is obtained as indicated by color change of indicator in culture solution. The air is freed of CO_2 and washed by passage through a soda lime column (*a*), 10-percent H_2SO_4 (*b*), and water (*c*). The CO_2 from culture flask (*d*) is aspirated into a series of tubes (*e*) containing standard $Ba(OH)_2$ solution.

The medium most commonly used in these investigations consisted of 2 g of $(NH_4)_2SO_4$, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4.7H_2O$, and 0.1 g of $Fe_2(SO_4)_3$ per liter of distilled water. Potato starch or glucose was usually used in concentrations of 2.5 percent. Ordinarily, the medium was distributed in Erlenmeyer flasks, but for respiration studies, round-bottom long-necked flasks were used. Incubation took place at 28° C. The cultures were allowed to remain undisturbed for 2 days; sterile $CaCO_3$ was then added, equal to the amount of carbohydrate added to the culture, and the flasks were shaken by hand, once or twice daily, for a minute or two. In the aeration studies, the alkali was added once every day and the culture well shaken during the addition of the alkali solution.

At the end of different periods of incubation, the cultures were removed and filtered through paper. The pellicles from the $CaCO_3$-free cultures were collected, dried at 100° C., weighed, and analyzed for total nitrogen and ash. When $CaCO_3$ was present in the medium, the pellicles were first washed with a dilute HCl solution and water. It was later found that an approximate determination of the growth of the organism could be obtained by washing the pellicle with hot water, analyzing for total nitrogen, and calculating the growth on the basis of the nitrogen in the pellicle. This calculation was especially useful for cultures containing calcium carbonate, which tended to give erroneously high weights. The filtrate and washings were made up to volume and analyzed for sugar by the Bertrand method, for ammonia by distillation, and for lactic acid by evaporation of an aliquot portion, drying, weighing, igniting, and reweighing. The lactic acid content was calculated from the amount of CaO or K_2O in the ash. This method of determining lactic acid is not strictly accurate, but it was regarded as being suitable for the experiments reported here, where, for the most part, marked changes in acid production were obtained as a result of the different treatments. Lactic acid is by far the chief acid produced by this organism; fumaric acid was found to be present in most cultures amounting up to 3 percent of the sugar consumed; small amounts of other unidentified acidic constituents were also found. Numerous analyses of the alcohol-precipitated salt from the cultures always yielded values for calcium content which checked closely with that for lactic acid.

The amounts of the other minerals left in the culture were negligible, having been largely assimilated by the pellicle. When ammonium sulphate was used as a source of nitrogen, allowance was made for the calcium sulphate produced as a result of the assimilation of the ammonia by the organism. The calcium sulphate production was found to be roughly parallel to the ammonia consumption. The latter was either calculated from the residual ammonia or from the total nitrogen in the pellicle.

In order to establish whether the acid thus produced by the fungus is primarily lactic, a quantity of the filtrate was evaporated to a small volume and the calcium lactate precipitated with alcohol. The precipitate was washed with alcohol, dried, weighed, and ashed. The CaO obtained corresponded closely with that commonly obtained from the calcium salt of lactic acid. The zinc salt was also prepared and analyzed for ZnO and for rotation. Here again the values checked closely with those for *d*-lactic acid. This procedure was repeated frequently during these experiments and always yielded similar results. When cultures were to be neutralized with KOH, a measured quantity of $CaCl_2$ was added before concentration in order to obtain the calcium salt. It was later found that upon the addition of alcohol, a small amount of a precipitate, consisting largely of phosphates and sulphates of calcium and magnesium, formed immediately. When this was rapidly removed by filtration and the liquid allowed to remain for 24 to 48 hours, the final precipitate was practically pure calcium lactate.

When starch was used as a source of carbohydrate, the residual starch of dextrin was determined by diluting an aliquot portion of the filtrate with water, making 2 percent acid by the addition of HCl, heating for 15 minutes at 15 pounds pressure, neutralizing, and deter-

mining the reducing sugar. Allowance was made for the sugar found directly in the culture.

GROWTH OF RHIZOPUS AND PRODUCTION OF LACTIC ACID

In the first experiment, 2 percent of raw potato starch was used as a source of carbohydrate. A large number of flasks were prepared so that several could be removed at different intervals. The total incuba-

TABLE 1.—*Carbohydrate decomposition and lactic acid production by Rhizopus and growth and nitrogen content of pellicle during incubation for various periods*

[Results on the basis of 1 liter of medium]

Period of incubation (days)	Starch [1] left, as sugar	Sugar present	Total carbohydrate consumed, as sugar	Lactic acid produced [2]	Total CO_2 liberated as C	NH_4 N consumed	Pellicle	
							Weight	Nitrogen content
	Milligrams	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*
0	18,920	0	0	0		0		
1	18,580	0	340	0				
2	17,000	540	1,380	0	100	17		
3	7,490	6,340	5,090	3,170	293	39	420	21
4	[3] 3,930	4,720	10,270	8,350	490	47	590	30
5	[3] 1,100	3,740	14,080	11,710	670	59		
7	[3] 400	1,280	17,240	14,720	941	73	[4] 740	37
8		300	18,620	14,820	1,163	82	[4] 920	46
10		60	18,860	13,340	1,522	78	1,090	56
12	0	0	18,920	12,770	1,710	94		
15	0	0	18,920		1,810	98	[4] 1,480	74
17	0	0	18,920	13,310	1,881	97		
24	0	0	18,920	13,220	1,946	95	[4] 1,780	89

[1] Including dextrin.
[2] Calculated from CaO in ash.
[3] No starch was left at 4 days; excess carbohydrate above sugar is due to dextrin.
[4] Calculated from nitrogen content.

tion period was 29 days. The results, presented in table 1, show that the starch was at first rapidly hydrolyzed by the organism to sugar, and only the sugar was utilized for lactic acid production. Within 4 days all the starch had disappeared, although it had not all been converted to sugar. A part of it was left in the medium in the form of dextrin and could be converted to sugar by acid treatment. The pellicle was found to contain approximately 5 percent of nitrogen.

During the early stages of growth of the organism, the sugar consumed, calculated as carbon, could not be fully accounted for by the sum of carbon in the pellicle, in the lactic acid, and in the carbon dioxide. After 3 days, for example, 5,090 mg of carbohydrate had disappeared, the equivalent of 2,036 mg of carbon. The three products accounted for only 1,719 mg of carbon, the carbon content of the pellicle being taken as 50 percent. Table 2 shows the carbon relationships at the different stages of development of the fungus.

TABLE 2.—*Carbon relationships at different stages of development of a fungus of the genus Rhizopus*

Period of incubation (days)	Carbohydrate consumed per liter of medium, as carbon	Carbon accounted for in lactic acid, pellicle, and CO_2	Period of incubation (days)	Carbohydrate consumed per liter of medium, as carbon	Carbon accounted for in lactic acid, pellicle, and CO_2	Period of incubation (days)	Carbohydrate consumed per liter of medium, as carbon	Carbon accounted for in lactic acid, pellicle, and CO_2
	Milligrams	*Milligrams*		*Milligrams*	*Milligrams*		*Milligrams*	*Milligrams*
	2,036	1,719	7	6,896	7,043	10	7,544	7,263
	4,108	3,973	8	7,448	7,383	24	7,568	7,984

The difference in the carbon balance during the early stages of growth is believed to have been due to the formation of an intermediary compound which was later converted to lactic acid, cell substance, CO_2, or to all of these. Subsequent experiments demonstrated that this was actually the case; volatile intermediary products were demonstrated.

The results of this experiment, and of other experiments reported previously (*29*), show that lactic acid production attains a maximum in 7 to 8 days, at 28° C.; a period which corresponds with that required for the complete disappearance of the carbohydrate. A detailed analysis of the course of growth of the fungus, consumption of glucose, production of lactic acid, and evolution of CO_2 is presented graphically in figure 2. The results of the previous experiment with starch as the substrate were fully confirmed.

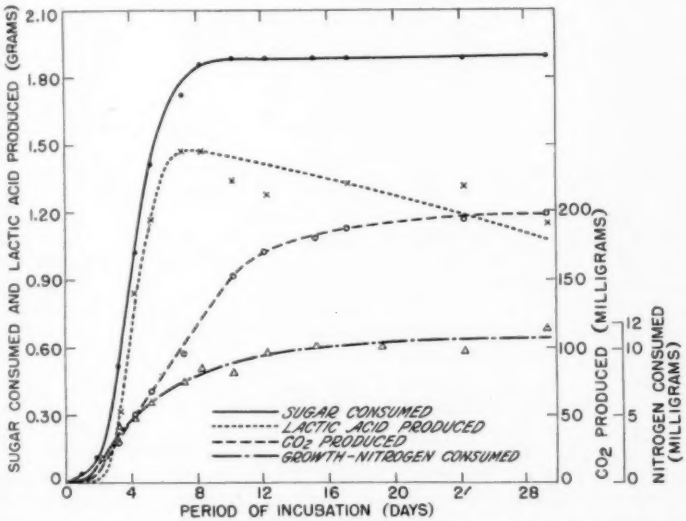

FIGURE 2.—Course of carbohydrate consumption, lactic acid production, CO_2 evolution, and growth of *Rhizopus*.

Assuming that lactic acid and CO_2 are the only final products of the reaction and leaving out of consideration the various intermediary products that are known to be formed, two possible explanations of the mechanism of lactic acid production by this fungus remain:

Skeleton reactions

(1) $$C_6H_{12}O_6 \rightarrow \underset{\text{(lactic acid)}}{C_3H_6O_3} + \underset{\text{(Unknown compound)}}{C_3H_6O_3};\ C_3H_6O_3 \xrightarrow{+3O_2} 3CO_2 + 3H_2O$$

(2) $$C_6H_{12}O_6 \xrightarrow{+3O_2} \underset{\text{(lactic acid)}}{C_3H_6O_3} + 3CO_2 + 3H_2O$$

In reaction (1), the ratio of $\frac{\text{lactic acid carbon}}{CO_2 \text{ carbon}}$ may be 1:1 or >1:1; the latter is possible when less than half of the lactic acid homologue is further oxidized. In reaction (2), the proportion of lactic acid carbon to CO_2 carbon cannot be greater than 1:1. By analyzing the results reported in table 1 for the 3-, 4-, 5-, 7-, 8-, and 24-day periods, the relations shown in table 3 are obtained.

TABLE 3.—*Analysis of the results presented in table 1 for the 3-, 4-, 5-, 7-, 8- and 24-day periods to present lactic acid-carbon dioxide ratios*

[Results on the basis of 1 liter of medium]

Period of incubation (days)	Carbohydrate C	Lactic acid C	CO_2C	$\frac{\text{Carbohydrate C}}{\text{(lactic acid C)}}$	$\frac{\text{Lactic acid C}}{(CO_2C)}$
	Milligrams	*Milligrams*	*Milligrams*		
3	2,040	1,220	290	1.7:1	4.2:1
4	4,110	3,190	490	1.3:1	6.5:1
5	5,630	4,580	670	1.2:1	6.8:1
7	6,900	5,730	940	1.2:1	6.1:1
8	7,450	5,760	1,160	1.3:1	5.0:1
24	7,570	5,290	1,950	1.4:1	2.7:1

The ratio of lactic acid C to CO_2C is always greater than 1:1. It ranges from 4.2:1 to 6.8:1, with an average for the five determinations during the early active stages of growth of 5.7:1. An analysis of the relation between carbohydrate consumed and lactic acid produced shows also a ratio greater than 1:1. These results point definitely to reaction (1) as the reaction concerned in the process of lactic acid production by *Rhizopus*. This reaction can be developed further to show that in addition to the lactic acid, an intermediary product is formed, which can be partly oxidized to CO_2 and partly transformed to lactic acid:

$$nC_6H_{12}O_6 = \underset{\text{Lactic acid}}{nC_3H_6O_3} + \underset{\text{Lactic acid}}{nC_3H_6O_3} + O_2 + 3CO_2 + 3H_2O$$

In the foregoing experiment, there was, after 24 days' growth, 5,290 mg of lactic acid carbon as compared with 1,950 mg of CO_2 carbon. At that time the ratio of carbohydrate carbon to lactic acid carbon was $\frac{7,570}{5,290}$, or 1.4:1, and the ratio of lactic acid carbon to CO_2 carbon was $\frac{5,290}{1,950}$, or 2.71:1. These ratios tend to approach the requirements for reaction (1). On the basis of the results of this experiment it may be concluded that *Rhizopus* breaks down the sugar first to some intermediate product, that part of this product is oxidized to CO_2, supplying the necessary energy for the growth of the organism, part is utilized for cell synthesis, and part is transformed to lactic acid. The balance between these three transformation products can be readily influenced by the conditions of growth of the organism. It will later be shown that the presence of different elements, notably salts of heavy metals, influence growth and lactic acid production differently.

In order to demonstrate quantitatively that the acid produced in the different stages of growth of the organism is lactic acid or predominantly so, the filtrates from several flasks of the organism grown on the above medium were collected after 7, 17, and 28 days' growth, concentrated on a water bath, and the calcium lactate precipitated with alcohol, washed with alcohol, and dried. The filtrate was further concentrated to a small volume and again precipitated with alcohol. The two precipitates were then combined and analyzed. The actual yields of calcium lactate obtained by precipitation were compared (table 4) with the amounts of lactic acid as calculated from the CaO content

TABLE 4.—*Purity and yield of acid produced by Rhizopus as compared with calculated yields after 7, 17, and 28 days of growth*

[Results on the basis of 1 liter of medium]

Item	7 days	17 days	28 days
	Milligrams	*Milligrams*	*Milligrams*
Total soluble material	17,852	18,120	16,220
Soluble ash	5,108	5,780	5,060
Minerals added to the medium [1]	1,850	1,770	1,680
Ash due to CaO	3,258	4,010	3,380
Lactic acid calculated from CaO	10,426	12,832	10,816
Sugar left in medium	3,263	0	0
Total soluble material accounted for [2]	17,886	17,466	14,910
Precipitation of soluble material with alcohol, total yield obtained	13,320	14,397	9,528
Total ash in precipitates	3,552	3,715	2,583
Ash calculated as Ca^{++}	2,573	2,653	1,845
Lactic acid by difference	10,747	11,744	7,683
	Percent	*Percent*	*Percent*
Ash found in lactate preparation [3]	26.7	25.8	27.1

[1] This is calculated from total minerals added minus the minerals in the ash in pellicle.
[2] Allowing for the CaO as Ca^{++} in the medium.
[3] Theoretical ash content in Ca lactate 25.7 percent; the slightly higher ash content found was later shown to be due to small amounts of phosphate impurities accompanying the lactate.

of the culture. After 7 days of growth of the organism, correlation between the two sets of results was very good. However, after 17 days, the yield of lactic acid by precipitation was somewhat less (8.5 percent) than that calculated from the ash content, and after 28 days the discrepancy was even greater (29.9 percent). Possibly this is due to the fact that, when all the sugar has disappeared, some of the lactic acid may become further oxidized to lower acids, as has been suggested previously (*7*, *11*, *29*).

INFLUENCE OF DIFFERENT FACTORS ON GROWTH OF RHIZOPUS AND PRODUCTION OF LACTIC ACID

REACTION OF THE MEDIUM

As pointed out above, the growth of *Rhizopus* upon carbohydrate media, as well as the formation and accumulation of lactic acid, is influenced considerably by the reaction and buffer content of the medium. In the following experiment the reaction was adjusted daily by the addition of sterile 0.5 N KOH solution. Some of the flasks were kept at pH 6.0, while others were made slightly alkaline, that is, pH 7.5 or higher. The results presented in table 5 show that when the reaction of the medium was kept alkaline, the organism produced more abundant growth but less lactic acid; in 12 days, there was more than twice as much growth at pH 7.5 as at pH 6.0, with about

half as much lactic acid produced. Thus conditions favorable to growth are unfavorable to lactic acid accumulation since more of the intermediary compound is used for energy, growth, and the metabolic activities of the organism. The nature of the acid formed was exactly the same when KOH was used as the neutralizing agent as when $CaCO_3$ was used.

TABLE 5.—*Influence of reaction of medium on growth of and lactic acid production by Rhizopus after 12 and 28 days*

[Results on the basis of 1 liter of medium]

Item	Medium pH 6.0		Medium pH 7.5+
	12 days	28 days	12 days
	Milligrams	*Milligrams*	*Milligrams*
NH_2N consumed	85	175	197
Fungus pellicle	1,680	2,670	3,790
N found in growth	61	142	178
Total soluble material	17,730	14,040	10,800
Soluble ash	7,540	5,640	5,040
Soluble K_2O	5,930	3,970	3,500
Lactic acid calculated from K_2O	11,360	7,600	6,700
Total soluble material accounted for	17,890	12,570	11,150
Lactic acid obtained by precipitation with alcohol as Ca salt	11,440	8,360	----------
Purified Ca $(C_3H_5O_3)_2$	9,600	----------	----------
	Percent	*Percent*	*Percent*
Ash in lactate	26.4	27.0	----------

In order to elucidate further the influence of reaction upon the metabolism of the organism, an experiment was made in which starch was used as the carbohydrate. In some of the flasks the reaction was adjusted daily to pH 5.5 and 6.5 by the use of sterile 0.25 N KOH solution; in the other flasks the reaction remained acid (about pH 4.0 or less). At the end of 4, 8, and 15 days, some of the flasks were removed for analysis. The organism began to grow rapidly at pH 4.0 (table 6), but in the absence of a neutralizing agent, the lactic acid

TABLE 6.—*Influence of reaction of medium on growth of and lactic acid production by Rhizopus in starch media after 4, 8, and 15 days*

[Results on the basis of 1 liter of medium]

Item	Medium pH, 4.0			Medium pH, 5.5			Medium pH, 6.5		
	4 days	8 days	15 days	4 days	8 days	15 days	4 days	8 days	15 days
	Milligrams	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*
Starch left as sugar	3,430	3,180	0	1,690	0	0	2,850	0	0
Sugar in medium	10,010	4,770	4,380	6,500	290	0	5,730	160	0
Carbohydrate consumed, as sugar	5,710	11,200	14,770	10,960	18,860	19,150	10,570	18,990	19,150
Growth of fungus	2,150	2,510	2,210	1,300	2,640	3,480	1,480	2,010	3,430
Nitrogen in growth	101	----------	93	73	134	168	71	108	176
Lactic acid produced [1]	----------	4,320	3,300	5,890	12,730	10,750	7,300	13,070	10,390

[1] Calculated.

soon made conditions unfavorable for further growth, by lowering the pH, so that, even after 15 days, considerable carbohydrate was left in the medium. At the less acid reaction growth was at first slower and was accompanied by more abundant lactic acid production. The

carbohydrate nearly disappeared in 8 days, but the organism continued to grow, presumably at the expense of the intermediary substance.

Three distinct steps are thus observed in the transformation of starch by *Rhizopus*. (1) The hydrolysis of the polysaccharide to sugar. This process is independent of the reaction of the medium within the pH range used and it depends entirely on the growth of the fungus. (2) The transformation of the sugar to lactic acid. This process is markedly influenced by the reaction of the medium. At a high acidity, only a limited amount of acid is formed, but the organism continues, to decompose the sugar, without producing any acid. Whether, under these conditions, the acid is formed and is again oxidized, or whether the sugar is oxidized directly remains to be determined. (3) Possible oxidation of the lactic acid to CO_2 through the stage of certain lower organic acids. This process has been observed only in old cultures, hence possibly it is not to be considered in the system of glucose dissimilation by the organism. As mentioned above, small quantities of fumaric acid were constantly found in these cultures. In this respect the organism behaves similar to *Rhizopus oryzae* (*11*). In the second step, the possibility is not excluded that, in addition to lactic acid, sugar gives rise to another intermediary product which is gradually oxidized further, before the lactic acid is itself attacked.

TEMPERATURE

Lockwood et al. (*11*) found that *Rhizopus* grew better at 40° than at 30° C., but that the yield of lactic acid and especially the efficiency of its production, or the ratio of glucose consumed to acid produced, were less at the higher temperature. A series of liter flasks containing 500-cc portions of 5-percent potato starch were inoculated with spores of the fungus and incubated at 28° for 48 hours. Sterile $CaCO_3$ was then added to all the flasks and these were incubated for 9 days at different temperatures. The flasks were shaken daily by hand, for a few minutes. The results (table 7) show that although the optimum

TABLE 7.—*Influence of temperature on growth of and lactic acid production by Rhizopus on potato-starch medium at different temperatures*

[Results on the basis of 1 liter of medium containing 50 g of raw potato starch]

Temperature (° C.)	Sugar left as as glucose	Lactic acid produced [1]	Weight of fungus pellicle
	Milligrams	*Milligrams*	*Milligrams*
20	9,100	24,900	2,800
28	3,400	34,200	3,200
37	400	30,300	3,600
45–50	29,100	5,400	300

[1] Calculated.

temperature for growth was 37°, that for acid production was 28°. Above and below these temperatures there was a rapid reduction in growth, an increase in sugar production from starch, and a decrease in lactic acid formation. Conditions unfavorable for the production or accumulation of lactic acid are not necessarily unfavorable for the formation of sugars from starch, a finding which again suggests a

difference in the mechanisms responsible for the processes of starch hydrolysis and glycolysis of sugar.

In a further experiment pellicles were employed for the study of the influence of temperature on the production of lactic acid from glucose. The fungus was grown at 28° C. in the presence of $CaCO_3$, the filtrate was removed, and the pellicles were washed with sterile water. Sterile glucose solution and sterile $CaCO_3$ were added to the flasks containing the pellicles, and these were incubated at different temperatures for 2 and 4 days. The results obtained (table 8) were similar to those of the preceding experiment (table 7).

TABLE 8.—*Influence of temperature on lactic acid production by Rhizopus pellicle on glucose medium at different temperatures*

[Results on the basis of milligrams per liter of medium containing 25 g glucose]

Temperature of incubation (° C.)	2 days' incubation		4 days' incubation	
	Sugar consumed	Lactic acid produced	Sugar consumed	Lactic acid produced
	Milligrams	*Milligrams*	*Milligrams*	*Milligrams*
20	11,430	----------	13,730	8,030
28	13,140	10,270	21,420	13,820
35	9,780	5,660	16,700	9,120
42	7,170	2,690	13,900	5,090
50	1,050	960	Trace	380

NITROGEN SOURCE

It has been shown (*29*) that nitrate is not a favorable source of nitrogen for the growth of *Rhizopus* or for lactic acid production, whereas ammonium salt is. Repeated comparisons between urea and ammonium sulphate as sources of nitrogen brought out the fact that both are favorable, provided precautions are taken not to split the urea to ammonium carbonate on sterilization, thus causing an alkaline reaction of the medium. In many of the subsequent experiments, urea was used as a source of nitrogen for the growth of the fungus, sterile urea solution being added to the medium. When fully grown pellicles were employed, the presence of available nitrogen was found to favor continued growth and to repress lactic acid accumulation.

RARE AND HEAVY METALS

The results of previous experiments brought out the fact that conditions favorable to the growth of the fungus are usually unfavorable to the production or accumulation of lactic acid. An attempt was made to find some chemical element or compound which would influence these two processes differently. The effect of salts of heavy metals and cell poisons was, therefore, studied. Records of a number of investigations are found in the literature concerning the action of heavy metals upon species of *Aspergillus*. According to McHargue and Calfee (*15*), the growth of *Rhizopus nigricans* is favored by the addition of Cu, Mn, and Zn, and especially by mixtures of these, in concentrations of 1 to 5 parts of the metal per million parts of medium. Lockwood et al. have also shown that zinc, in concentrations of 10 to 50 mg per liter, stimulates the growth of *Rhizopus* and the consumption of glucose; however, the presence of this element was found to result in a reduction in the yield of lactic acid.

In the present studies, the basic medium consisted of 2 percent of glucose, 0.1 percent of $(NH_4)_2SO_4$, 0.05 percent of K_2HPO_4, and 0.05 percent of $MgSO_4.7H_2O$. The salts of the various elements tested were added on an anhydrous basis. Technical glucose was employed in the first experiment and c. p. anhydrous glucose in the others. In order that the effect of an organic source of nitrogen might be compared with that of the inorganic salt, 0.2 percent of peptone was substituted for the ammonium salt in one series of flasks. Some of the flasks received $CaCO_3$ in excess (20 g per liter) and the others were left free of carbonate. Analyses were made in duplicate after 7, 11, and 25 days' incubation at 28° C. and in a few cases after 4 days.

TABLE 9.—*Influence of heavy metals and certain rare elements, in the presence and in the absence of calcium carbonate, on the growth and acid production by Rhizopus at different stages of development*

[Results on the basis of 1 liter of medium]

Treatment	Growth in [1]—			Glucose consumed in—		Nitrogen consumed in—		Lactic acid produced in [2]—			
	7 days	11 days	25 days	4 days	7 days	7 days	11 days	4 days	7 days	11 days	25 days
Calcium carbonate used	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*
Control	2,100	2,030	1,950	.....	17,840	77	93	.....	13,550	10,440	10,660
$FeCl_3$	1,580	1,570	2,180	4,420	17,080	55	60	5,390	14,140	13,920	8,400
$ZnSO_4$	4,520	3,620	3,750	13,170	18,360	185	176	5,890	7,750	5,770	3,600
$MnSO_4$	1,950	2,070	.....	.....	17,290	72	91	.....	13,270	12,930	.....
$CuSO_4$	2,150	.....	.....	1,560	16,180	79	.....	Tr.	11,560	.....	.....
Na_3BO_3	2,050	2,150	.....	.....	17,630	70	102	.....	15,930	10,700	.....
Na_2MoO_4	2,250	2,150	2,070	.....	17,790	82	97	.....	12,740	10,790	9,890
Peptone [3]	2,030	1,900	2,000	.....	17,950	81	[4] 91	.....	15,160	11,720	12,250
No calcium carbonate											
Control	2,000	.....	.....	.....	12,840	91	.....	.....	.....	.....	.....
$FeCl_3$	1,480	.....	.....	5,910	9,690	85	.....	5,330	.....	.....	.....
$ZnSO_4$	3,500	.....	2,790	10,810	14,480	185	.....	2,570	.....	.....	5,670
$MnSO_4$	2,420	.....	.....	.....	11,260	110	.....	.....	.....	.....	.....
$CuSO_4$	2,490	.....	.....	4,320	11,010	117	.....	3,470	.....	.....	.....
Na_3BO_3	1,430	.....	.....	.....	11,440	62	.....	.....	.....	.....	.....
Na_2MoO_4	1,820	1,580	.....	.....	12,710	77	94	.....	.....	.....	.....
Peptone	1,950	1,530	1,730	.....	12,480	[4] 74	[4] 68	.....	.....	.....	3,350

[1] The pellicle in the $CaCO_3$ flasks was calculated from the nitrogen content, as found in the pellicles grown in the $CaCO_3$-free flasks, to avoid washing with HCl.
[2] Calculated from calcium in solution.
[3] In place of NH_4 as source of nitrogen.
[4] Nitrogen actually found in pellicle.

The results of the first experiment (table 9) show that the salts of various heavy metals and of other elements not commonly employed in culture media had a different effect on the growth and lactic acid production of *Rhizopus*. Some elements, such as iron and copper, had a depressing effect, in the concentrations used, both on growth and glucose consumption, but the effect on acid production was favorable. Other elements, especially zinc, were highly stimulating to the growth of the organism and to sugar consumption, but the effect on acid accumulation was depressive. Certain elements, such as manganese and molybdenum, had little additional effect on either growth or acid formation. Boron stimulated acid production at first, but later this effect tended to disappear. Peptone had no advantage over the ammonium salt as a source of nitrogen and con-

tributed none of the so-called biotic factors, for which this organism has no particular need. The results of this experiment point definitely to the presence of certain elements, notably zinc, as favoring growth of the fungus. Growth, however, takes place at the expense of the formation of lactic acid.

Further experiments were performed in which various concentrations of some of the above elements, as well as combinations of these, were used in order to determine whether both maximum growth and maximum acid production can take place simultaneously. Anhydrous c. p. glucose (2.5 percent) was used in this experiment; 0.2 percent of $(NH_4)_2SO_4$, 0.1 percent of K_2HPO_4, and 0.05 percent of $MgSO_4.7H_2O$ were added to make up the stock medium; the salts of the various elements were calculated on an anhydrous basis. All the flasks received $CaCO_3$, 2 days after inoculation.

TABLE 10.—*Influence of heavy metals, and certain rare elements in different concentrations on the growth and acid production by Rhizopus after 5, 11, and 18 days*

[Results on the basis of 1 liter of medium]

Treatment [1]	Growth in—			Glucose consumed	Nitrogen consumed in—			Lactic acid produced in—		
	5 days	11 days	18 days	5 [2] days	5 days	11 days	18 days	5 days	11 days	18 days
No Fe, no Zn	2,450	2,950	3,090	19,620	104	123	126	13,580	13,700	13,800
Fe:										
2 mg	2,420	3,380	3,160	18,340	97	129	129	12,960	12,960	13,390
5 mg	2,270	3,070	2,870	18,350	85	117	126	13,550	13,670	14,040
20 mg	1,270	2,310	2,530	14,070	41	87	117	12,400	15,280	14,070
Zn:										
5 mg	5,920	6,380	5,620	20,810	305	313	261	6,050	7,810	8,840
20 mg	5,000	6,820	-------	20,490	258	337	-------	7,560	5,860	-------
5 mg Fe+5 mg Zn	6,970	7,020	7,400	20,880	361	370	379	2,760	4,340	3,160
5 mg Fe+10 mg Zn	6,200	8,020	7,420	19,460	332	379	379	3,940	4,030	5,020
5 mg Fe+5 mg Zn+5 mg Mn	6,700	8,330	8,180	20,630	358	379	379	2,910	3,660	4,090
5 mg Fe+5 mg Zn+5 mg Mn +5 mg Cu	6,800	8,030	8,140	21,100	370	379	379	2,640	3,350	2,670
Na_3BO_3, 5 mg	1,770	-------	2,650	18,180	72	-------	123	13,760	-------	14,350
Na_3BO_3, 10 mg	2,010	-------	2,650	18,410	92	-------	117	12,960	-------	13,890

[1] Fe=$Fe_2(SO_4)_3$, Zn=$ZnSO_4.7H_2O$, Mn=$MnSO_4.5H_2O$, Cu=$CuSO_4.5H_2O$, all on an anhydrous basis; milligrams of salt per liter of medium.

[2] All sugar (24,050 mg) consumed in 11 days.

Zinc was again found (table 10) to be specific in catalyzing the growth of the fungus. None of the other elements tested could take its place; they merely supplemented it by stimulating growth further. The effect of iron upon growth was, by itself, limited; however, when zinc was also added, there was an increased stimulating effect. The same was true of manganese and possibly copper. The influence of the last three elements was exerted directly upon the growth of the fungus (fig. 3). In all cases increased growth was accompanied by a reduction in the amount of lactic acid produced. This suggests the possibility that these elements favor oxidation of the precursor of lactic acid, thus resulting in a decrease in the formation of this acid, and thereby making more energy available for the growth of the fungus. In order to determine whether the organism is capable of oxidizing the lactic acid itself, an attempt was made to grow it on a calcium lactate medium, with and without the zinc salt. It was

found that neither spore inoculation nor young pellicles made any growth with lactic acid as the sole source of energy.

The following experiment illustrates further the specific effects of zinc on the growth and acid production of *Rhizopus*, as well as on the lactic acid formed. Eight liters of medium containing 5 percent of c. p. glucose and nutrient salts were divided into two lots, one receiving 0.001 percent of $Fe_2(SO_4)_3$ and the other 0.001 percent of $ZnSO_4$. The media were distributed, in 400-cc portions, into 1-liter flasks, sterilized, and inoculated. Ten-gram portions of $CaCO_3$ were added to some of the flasks. These were incubated at 28° C. and analyzed in duplicate after 6, 11, 16, and 25 days. The filtrates were evaporated to a small volume, precipitated with alcohol, and the lactate recovered.

The results presented in table 11 show that, although in the presence of zinc the sugar was consumed more rapidly, the growth of the fungus was even more rapid. After 6 days' incubation, the consumption of the sugar was, in favor of the zinc, 38.47: 26.05 or a ratio of 1.48:1, but, the corresponding dry weights of the fungus pellicle for the same period were 4.78:1. This extensive development of the fungus took

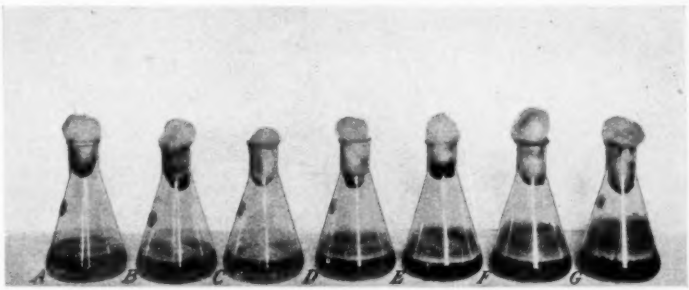

FIGURE 3.—Influence of different elements upon the growth of *Rhizopus*, on liter basis: *A*, Control: *B*, 2 mg $Fe_2(SO_4)_3$; *C*, 5 mg $Fe_2(SO_4)_3$; *D*, 5 mg $ZnSO_4$; *E*, 10 mg $Fe_2(SO_4)_3$ + 10 mg $ZnSO_4$; *F*, 5 mg each of $Fe_2(SO_4)_3$, $ZnSO_4$, and $MnSO_4$; *G*, 10 mg each of Fe, Zn, Mn, and Cu (as $CuSO_4$) salts.

place at the expense of the formation of lactic acid, which was reduced to less than one-half in the presence of zinc as compared with that in cultures not containing zinc. Similar results were obtained after 11, 16, and 22 days, when all the sugar had disappeared both in the presence and in the absence of zinc. The growth of the fungus was still twice as great in the presence of zinc as in its absence and the production of lactic acid was greatly reduced. The nitrogen was also consumed more rapidly in the cultures with zinc, so that additional ammonium salt had to be introduced (250-mg portions per flask), after 7 and 12 days' incubation.

The results of another experiment on the decomposition of glucose, in the presence of $CaCO_3$, are presented graphically in figure 4. In the absence of zinc, lactic acid was rapidly produced in the cultures of *Rhizopus* and was allowed to accumulate. At first the acid yield was high; then it gradually lessened up to about 13 days, when it remained constant. In the presence of zinc, the yield of lactic acid was considerably lower; however, when maximum growth was attained, lactic acid began to accumulate. It is of particular interest to note that, during

TABLE 11.—*Influence of iron and zinc upon the sugar and nitrogen consumed and the lactic acid produced by Rhizopus, and on its growth, in the presence and in the absence of calcium carbonate*

[Results on the basis of 1 liter of medium containing 5 percent of anhydrous glucose]

Period of incubation (days)	$CaCO_3$ added	Sugar consumed		Nitrogen consumed		Growth of fungus		Lactic acid calculated from CaO		Lactic acid actual yield	
		Fe	Zn	Fe	Zn	Fe	Zn	Fe	Zn	Fe	Zn
		Grams	*Grams*	*Milligrams*	*Milligrams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*
6	+	26.05	38.47	97	336	1.38	6.60	15.45	7.43	11.50	7.06
11	+	42.00	46.20	170	486	3.83	9.03	21.81	8.55	18.26+	6.83+
16	+	46.20	46.20	224	495	5.08	10.36	24.21	9.83	16.00+	6.14+
22	+	46.20	46.20	233	494	5.57	11.78	22.64	9.16	17.98	6.61
11	−	15.07	13.64	139	236	2.29	3.33	Tr.	Tr.	.....	.....
22	−	19.57	18.87	192	239	2.64	3.01	Tr.	Tr.	.....	.....

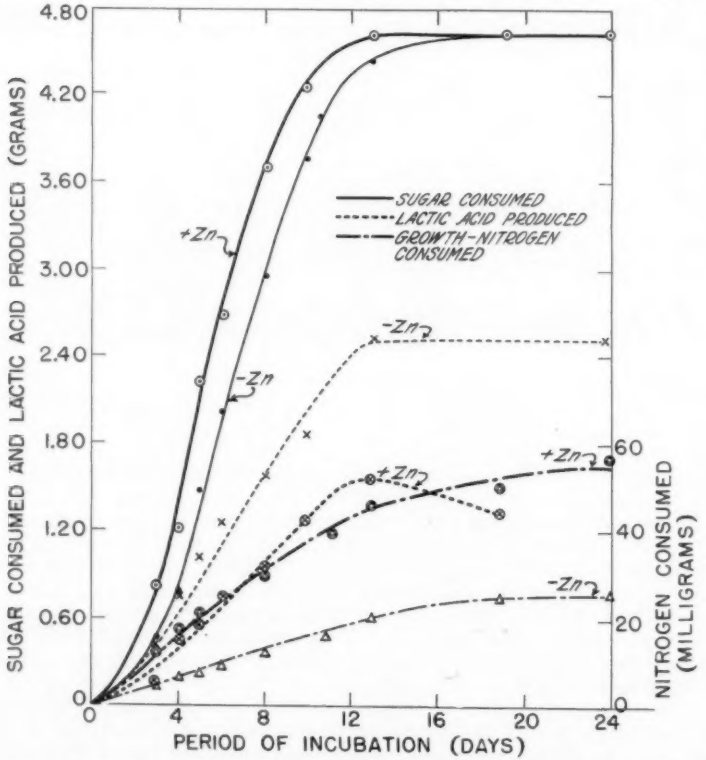

FIGURE 4.—Course of sugar decomposition, growth (nitrogen consumption), and lactic acid production by *Rhizopus*, as influenced by the presence of zinc in the medium.

the early stages of growth of the fungus, there was an inverse parallelism between growth and lactic acid production, both in the absence and in the presence of zinc. These results tend to show that zinc favors a more abundant growth of the organism, which is accompanied by a more rapid and complete destruction of the sugar, while lactic acid does not accumulate. In the absence of zinc, the carbohydrate is transformed largely to the lactic acid, with the liberation of a smaller amount of energy, as a result of which growth of the fungus is much more limited.

INFLUENCE OF OXYGEN TENSION ON LACTIC ACID PRODUCTION BY FUNGUS PELLICLES

To obtain further information concerning the process of lactic acid production by *Rhizopus*, it was necessary either to eliminate the growth of the organism or to reduce it as much as possible. This could be accomplished by the use of fully developed pellicles. The fungus was grown in 200-cc portions of medium placed in 500-cc Erlenmeyer flasks. The medium contained, in addition to the ordinary minerals, 2.5 percent of glucose and 0.001 percent of $ZnSO_4$, and to obtain more vigorous growth $CaCO_3$ was usually added also. After 6 to 7 days' incubation at 28° C., the filtrate was removed and the pellicle washed thoroughly with sterile distilled water. Sterile portions of sugar solution were then added to the flasks containing the pellicles.

TABLE 12.—*Influence of oxygen tension on lactic acid production by Rhizopus pellicles, in the presence and in the absence of calcium carbonate, after 4, 7, 10, and 15 days of growth*

[Results on the basis of 1 liter of medium]

Item	4 days				7 days				10 days			15 days	
	Aerobic		Anaerobic		Aerobic		Anaerobic		Aerobic	Anaerobic		Aerobic	Anaerobic
	$CaCO_3$+	$CaCO_3$−	$CaCO_3$+	$CaCO_3$−	$CaCO_3$+	$CaCO_3$−	$CaCO_3$+	$CaCO_3$−	$CaCO_3$−	$CaCO_3$+	$CaCO_3$−	$CaCO_3$−	$CaCO_3$−
	G.	*G.*	*G.*	*G.*	*G.*	*G.*	*G.*	*G.*	*G.*	*G.*	*G.*	*G.*	*G.*
Sugar consumed	16.73	13.85	17.62	15.00	25.00	17.35	23.23	20.46	18.65	25.00	23.40	22.61	22.64
Free lactic acid	.42	4.32	.64	3.94	.15	5.28	.29	6.66	7.01	.29	6.15	7.84	6.00
Lactic acid as calcium salt	8.91	.07	7.41	1.17	14.79	.29	11.71	.55	0	12.29	1.63	.48	1.06
Total lactic acid	9.33	4.39	8.05	5.11	14.94	5.57	12.00	7.21	7.01	12.58	7.78	8.32	7.06
	Per-cent	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*
Sugar converted to lactic acid	56	32	46	34	60	32	52	35	38	50	33	37	31

The results of the first experiment (table 12) show that, under aerobic conditions and in the presence of $CaCO_3$, the pellicle of the fungus converted 56 percent of the sugar to lactic acid in 4 days and 60 percent in 7 days. In the absence of $CaCO_3$ the rate of glycolysis was slower and the yield of acid less. The small amount of acid reported as the calcium salt when no $CaCO_3$ was added was due to some carbonate adhering to the pellicle which was not removed by the washing process. The rate of glycolysis, as measured by the disappearance of the sugar, was practically the same under anaerobic as

under aerobic conditions, but the rate of lactic acid production was less under anaerobic conditions, usually about 50 percent, in the presence of $CaCO_3$. When the CO_2 liberated under anaerobic conditions and in the absence of $CaCO_3$ was collected and measured, it was found to be approximately equivalent to the amount that would be expected from reaction (1), i. e., equimolecular concentrations of sugar consumed.

These results prove conclusively that lactic acid formation can take place under anaerobic conditions, by a fermentative mechanism. They prove further that the presence of oxygen does not hinder the production of lactic acid but may actually favor it, although the production process is accompanied by the consumption of some of the energy and carbon for growth purposes. These results suggest further that the enzyme system of the pellicle transforms the carbohydrate to lactic acid and to some other unidentified substance of the nature of an alcohol. This was later shown to be ethyl aclohol, by formation of the dinitro-benzoic acid ester. Under aerobic conditions this alcohol or its precursor is oxidized further, yielding energy for growth and giving more lactic acid.

The above experiment was repeated with pellicles grown for 8 days on a urea–$CaCO_3$ medium (containing also glucose, $ZnSO_4$ and other minerals). The pellicles were washed and sterile 2.5-percent glucose solution added. Some of the flasks were thoroughly aerated by bubbling air through the culture 5 to 8 hours every day, while others were kept in a CO_2 atmosphere. After 5 days' incubation at 28° C., all the flasks were removed for analysis. The results obtained (table 13) confirmed those of the previous experiment in showing that under anaerobic conditions and in the presence of $CaCO_3$, only 50 percent of the carbohydrate was converted to lactic acid, while in the presence of an excess of oxygen more lactic acid was formed.

TABLE 13.—*Influence of aeration on lactic acid production by Rhizopus pellicles in the presence and in the absence of calcium carbonate*

[Results on the basis of 1 liter of medium]

Item	Aerated		Anaerobic	
	$CaCO_3$	No $CaCO_3$	$CaCO_3$	No $CaCO_3$
	Grams	*Grams*	*Grams*	*Grams*
Sugar consumed [1]	26.70	26.70	23.56	24.37
Free lactic acid		7.00		7.81
Lactic acid as calcium salt	15.36	4.06	12.12	2.61
Total lactic acid	15.36	11.06	12.12	10.42
	Percent	*Percent*	*Percent*	*Percent*
Sugar converted to lactic acid	57.5	41.4	51.5	42.8

[1] Sugar in control was 26.70 g.

The pellicles were also found to be capable of converting starch to lactic acid. In the following experiment a sterile 2.5-percent solution of raw starch was added to 7-day-old pellicles grown on glucose + $CaCO_3$ medium. No nutrients were added to the starch solution. The flasks were incubated at 28° C. under anaerobic conditions for 7 days (table 14). All the starch disappeared (iodine test) and only a small amount of reducing sugar was left. The amount of starch trans-

formed to lactic acid was also about 50 percent, in the presence of $CaCO_3$. No volatile acid was formed except in mere traces as a result of the action of the pellicle on glucose and starch. However, large amounts of ethyl alcohol were demonstrated, especially in the anaerobic cultures. The formation of this alcohol accounts, together with the CO_2, for that part of the carbohydrate which was not converted to lactic acid.

TABLE 14.—*Lactic acid production from starch by pellicles of Rhizopus, in the presence and in the absence of calcium carbonate*

[Results on the basis of 1 liter of 2.5-percent starch solution [1]]

$CaCO_3$ added	Sugar left	Starch, as sugar, consumed	Free lactic acid	Combined lactic acid	Total lactic acid
	Grams	*Grams*	*Grams*	*Grams*	*Grams*
(−)	2.29	22.59	7.70	2.21	9.91
(+)	.87	24.01	0	13.12	13.12

[1] Total starch in 1 liter of medium equivalent to 24.88 g of reducing sugar.

The influence of nitrogen and minerals on glycolysis of sugar by the fungus pellicle is brought out in table 15. The data there shown emphasize the fact that the addition of available nitrogen favors the further growth of the pellicle and results in a depressing effect upon lactic acid production; however, the addition of minerals only, without the nitrogen, favored increased production of the acid.

TABLE 15.—*Influence of nitrogen and minerals on lactic acid production by Rhizopus pellicles* [1]

[Results on the basis of 1 liter of medium]

Item	Treatment		
	Urea [2]	Minerals [3]	Urea + minerals
	Grams	*Grams*	*Grams*
Sugar consumed	25.82	25.16	24.99
Lactic acid formed	11.23	17.66	10.13
	Percent	*Percent*	*Percent*
Sugar converted to acid	43.5	70.2	40.5

[1] Pellicles similar to those used in previous experiment (table 14) were employed; all flasks received $CaCO_3$; flasks kept stationary, with oxygen admitted.
[2] 0.1 percent.
[3] Equivalent to those added to medium.

By increasing the concentration of glucose, a single pellicle weighing approximately 1 g, on a dry basis, was found to be able to convert completely 40 g of glucose, in the presence of $CaCO_3$, to lactic acid and alcohol. In the presence of 20 percent of glucose, sufficient lactic acid was formed to solidify the medium because of the crystallization of the calcium lactate. The relation between glucose concentration and time of action of pellicle is brought out in figure 5. The proportional relationship definitely points to an enzyme mechanism. Concentrations of glucose above 20 percent had a depressing effect on glycolysis and resulted in the autolysis of the fungus cells.

The results of these and of other experiments, not reported here, lead to the following conclusions: (1) The presence of nitrogen and mineral nutrients is not necessary, in the case of fungus pellicles, for active lactic acid production to set in immediately; (2) the rate of formation of lactic acid by the pellicle depends on its age and vigor, rather than on the nature of the medium in which it is grown; (3) $CaCO_3$ is not necessary for the production of lactic acid by the pellicle, although more of the acid accumulates in the presence of the carbonate than in it absence; (4) there is no difference in the nature of the acid produced during the growth of the organism or that produced by the

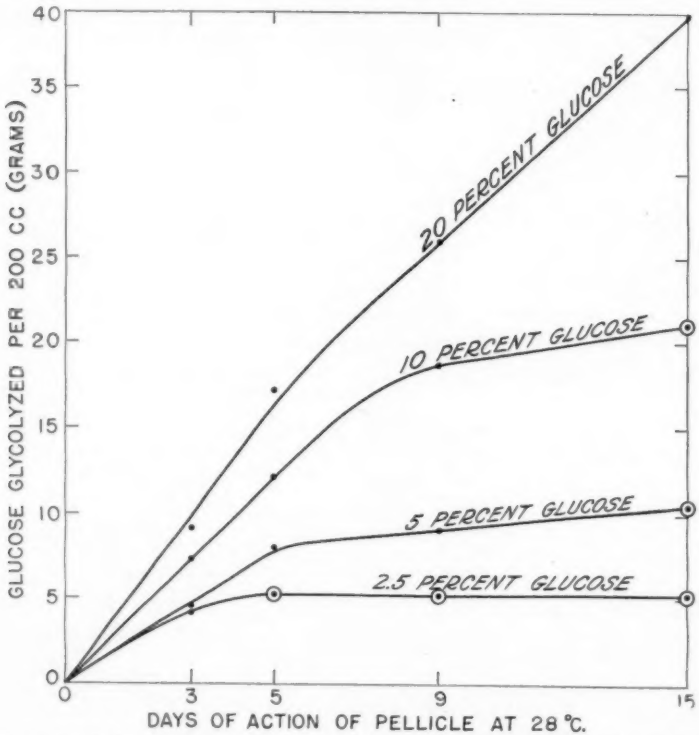

FIGURE 5.—Influence of glucose concentration on the rate of glycolysis by *Rhizopus* pellicles. Circles indicate that all the sugar has disappeared.

action of the pellicles on sugar or starch solution; (5) the amount of lactic acid produced by the pellicles acting on sugar depends to a certain extent on the oxygen tension, more lactic acid being formed under aerobic conditions; (6) under anaerobic conditions, abundant formation of ethyl alcohol takes place; (7) the fungus pellicle does not continue to grow under anaerobic conditions, whereas some growth occurs under aerobic conditions, especially when air is passed through the cultures and when additional nitrogen is present.

LACTIC ACID PRODUCTION FROM DIFFERENT CARBOHYDRATES AND RELATED COMPOUNDS BY RHIZOPUS PELLICLES

In all the previous experiments, glucose and starch were used as sources of carbon. In order to compare the production of lactic acid from other organic compounds by the fungus pellicle, a variety of sugars and sugar alcohols were selected. The organism was grown for 7 days upon a glucose solution containing the necessary nutrients and $CaCO_3$. The filtrate was removed, the pellicles were washed and the various compounds added, in concentrations of 2.0 to 2.5 percent, in the form of sterile solutions. All the flasks received $CaCO_3$ and were incubated at 28° C., at ordinary atmospheric pressure. The results, presented in table 16, show that active lactic acid formation by the fungus pellicle took place from glucose, maltose, dextrin, and levulose, and to a lesser extent from galactose and xylose. Although the organism could utilize lactose, rhamnose, and mannitol for growth, it produced no lactic acid from them. Attention may be called to the fact that in 1911 Saito (*27*) reported that *Rhizopus chinensis* produced lactic acid from glucose, levulose, maltose, and dextrin, but not from sucrose, inulin, or lactose.

TABLE 16.—*Lactic acid production by Rhizopus pellicles from different carbohydrates and related compounds after 3 and 7 days of growth*

[Results on the basis of 1 liter of medium]

Source of carbon	Reducing sugar consumed in—		Lactic acid produced—		Source of carbon	Reducing sugar consumed in—		Lactic acid produced—	
	3 days	7 days	3 days	7 days		3 days	7 days	3 days	7 days
	Grams	*Grams*	*Grams*	*Grams*		*Grams*	*Grams*	*Grams*	*Grams*
Glucose	14.35	19.62	5.56	8.45	Rhamnose		4.30		Tr.
Maltose	12.27	19.48	6.85	9.54	Lactose	0.4	2.51	0	Tr.
Dextrin	15.65	22.37	6.75	9.06	Sucrose [1]	0	0	0	Tr.
Arabinose	.14	3.54	Tr.	Tr.	Inulin	0	0	0	Tr.
Xylose	7.32	8.04	1.38	2.14	Mannitol [2]	1.48	3.13	0	Tr.
Galactose	10.91	11.12	2.62	4.54	Glycerol [2]	0	0	Tr.	Tr.
Levulose	14.45	22.55	9.15	10.21					

[1] On hydrolysis with dilute acid.
[2] Soluble material.

INFLUENCE OF POISONS ON GLYCOLYSIS BY RHIZOPUS PELLICLE

In order to determine the chemical nature of the break-down of the sugar by the fungus, a study was made of the influence of respiration poisons on the glycolytic effect of the pellicle. Substances known to repress the action of the glycolytic process, namely, sodium fluoride and iodoacetic acid, were utilized. Several 6-day-old pellicles were washed with water and 2.5-percent sterile glucose solution added to them. Different concentrations of the two poisons in sterile solutions were added. Some of the flasks received $CaCO_3$ and others did not. Incubation took place at 28° C. for 6 days in an anaerobic atmosphere. The results, presented in table 17, show that NaF, in concentrations of 0.02 to 0.10 percent, had only a limited effect on glycolysis and lactic acid production; these two processes were not repressed even with the highest concentrations of the poison. The iodoacetic acid repressed glycolysis in a concentration of 0.02 percent but not in the

lower concentrations used. The CO_2 liberated by the action of the pellicles was somewhat repressed by the poisons, especially in the higher concentrations. The larger amount of CO_2 produced in the presence of $CaCO_3$ was due to chemical interaction of the latter with the lactic acid.

A study was made of the influence of various concentrations of iodoacetic acid on the production of lactic acid by fully grown pellicles. These were obtained by growing the organism, for 6 days, upon a urea – $CaCO_3$–$ZnSO_4$ medium. The pellicles were washed with water and a 2.5-percent glucose solution was added. The flasks were incubated for 5 days at 28° C. in the presence of oxygen. Because of the short incubation period, the recovery of the acid was not complete. The results of this experiment (table 18) show again that increasing concentrations of the iodoacetic acid repressed both sugar glycolysis and lactic acid production, the latter to a much greater extent than the former. The pellicle glycolyzed the sugar even in the presence of 0.1 percent of the poison, but under similar conditions less than 10 percent of the sugar was recovered as lactic acid. This was true both when the reaction was neutral and when it was acid.

TABLE 17.—*Influence of different concentrations of glycolytic poisons on lactic acid production by Rhizopus pellicles in the presence and in the absence of calcium carbonate*

[Results on the basis of 1 liter of sugar solution]

Poison used	Concentration of poison	$CaCO_3$	Sugar consumed	Lactic acid produced	CO_2 produced (as C) [1]
	Percent		*Grams*	*Grams*	*Milligrams*
None	0.00	−	17.73	8.83	346
	.00	+	20.92	9.79	617
Sodium fluoride	.02	−	8.76	2.83	337
	.02	+	16.13	4.80	463
	.04	−	6.86	2.93	246
	.04	+	15.42	5.38	309
	.10	−	5.16	2.40	215
	.10	+	16.67	8.35	321
Iodoacetic acid	.004	−	9.00	2.45	375
	.004	+	11.94	6.82	552
	.008	−	8.84	4.01	305
	.008	+	11.79	5.67	374
	.02	−	4.11	.77	113
	.02	+	7.74	2.11	247

[1] The pellicle alone gave off, in an aqueous solution, 192 mg of carbon as CO_2, in the absence of $CaCO_3$, and 156 mg of carbon in the presence of $CaCO_3$.

TABLE 18.—*Influence of different concentrations of iodoacetic acid on sugar consumed and lactic acid produced by Rhizopus pellicles in the presence and in the absence of calcium carbonate*

[Results on the basis of 1 liter of sugar solution]

Concentration of iodoacetic acid (percent)	$CaCO_3$	Sugar consumed	Lactic acid produced	Sugar converted to lactic acid	Concentration of iodoacetic acid (percent)	$CaCO_3$	Sugar consumed	Lactic acid produced	Sugar converted to lactic acid
		Grams	*Grams*	*Percent*			*Grams*	*Grams*	*Percent*
0.00	+	23.4	9.2	39.3	0.00	−	22.6	5.9	26.1
.01	+	19.0	5.2	27.4	.01	−	17.8	4.3	24.2
.02	+	15.1	2.8	18.5	.02	−	13.7	2.6	19.0
.04	+	13.1	1.8	13.7	.04	−	12.7	1.2	9.4
.10	+	10.6	.9	8.5	.10	−	10.5	.7	6.7

An experiment was made to determine the effect of iodoacetic acid on the growth of the fungus. The regular ammonium sulphate-glucose-$ZnSO_4$ medium containing the different concentrations of the poison was inoculated with spores of the fungus and incubated for 6 days. Calcium carbonate was added only to those flasks that showed active growth of the fungus. The results (table 19) show that con-

TABLE 19.—*Influence of different concentrations of iodoacetic acid upon the growth of Rhizopus from spores*

[Results on the basis of 1 liter of medium]

Concentration of iodoacetic acid (percent)	Sugar consumed	Lactic acid produced	Concentration of iodoacetic acid (percent)	Sugar consumed	Lactic acid produced
	Grams	*Grams*		*Grams*	*Grams*
0.00	25.8	9.90	0.03	0.9	0.2
.01	24.3	9.3	.10	.3	0

centrations of iodoacetic acid greater than 0.01 percent in the medium completely represses the growth of *Rhizopus*.

FORMATION OF INTERMEDIARY PRODUCTS IN THE GROWTH OF RHIZOPUS

The results of the foregoing experiments have established definitely that nutrition and energy utilization of *Rhizopus* do not take place through the lactic acid stage, as originally assumed. Actually the lactic acid is a byproduct of the reaction and can be oxidized further by the organism only to a very limited extent, if at all. It is to the other product of the break-down of the sugar that one must look for the energy and carbon source of the organism. This product is ethyl alcohol; it is formed in the anaerobic break-down of the sugar; under aerobic conditions, it is either not formed at all or further oxidized. It may, of course, be assumed that the alcohol arises through the usual pyruvic acid stage; however, it is the alcohol which is the intermediary between the anaerobic and the aerobic phases of the respiration process. In other words, we have here a good example of the Pasteur reaction. This is clearly illustrated by the results of an experiment on the formation of alcohol by *Rhizopus* pellicles under aerobic and anaerobic conditions (table 20). Under anaerobic conditions the concentrations of lactic acid and of ethyl alcohol produced were practically equimolecular with the glucose consumed. Under aerobic conditions, however, the amount of alcohol found was very small and the lactic acid produced was much greater than the equimolecular concentration. Whether the excess lactic acid is produced from the alcohol or from its precursor still remains to be determined. The fact is that the organism is able to utilize ethyl alcohol readily as a source of energy and carbon.

TABLE 20.—*Glucose consumed and lactic acid and ethyl alcohol produced by Rhizopus pellicle under aerobic and anaerobic conditions*

[Results on the basis of 1 liter of medium]

Period elapsed (days)	Aeration	Glucose consumed		Lactic acid produced		Ethyl alcohol produced	
		Grams	*Millimols*	*Grams*	*Millimols*	*Grams*	*Millimols*
15	Anaerobic	53.9	300	28.5	316	13.1	286
20	do	57.3	318	29.4	327	15.8	344
15	Aerobic	82.8	460	61.5	684	4.3	94

DISCUSSION

Three distinct factors or groups of factors are primarily concerned in the growth of micro-organisms upon natural and artificial substrates, and to a large extent they control the formation and accumulation of intermediary and waste products of microbial metabolism. These factors are (1) biological, including the nature of the organism, its strain specificity, and its previous cultivation; (2) nutritional, comprising the nature of the energy source and its concentration, the nature and concentration of the nitrogen source, the nature and abundance of other nutritive elements, and the presence of certain special elements that have a positively or a negatively stimulating effect on the growth of the particular organism and on its specific metabolism; (3) environmental, including temperature, reaction, oxygen tension, length of cultivation, etc.

In the study of the physiology of the lower fungi, these factors were found not only to influence the quantitative accumulation of certain metabolic products but to modify the very nature of these products. It is sufficient to call attention to the preferential formation of gluconic acid or of citric and oxalic acids by *Aspergillus niger;* of ethyl alcohol or of glycerol by yeasts; of butyl alcohol or of butyric acid by certain anaerobic bacteria; of lactic acid or of acetic acid by acetic acid bacteria, etc. The results of the investigations reported in this paper on the mechanism and conditions of formation of lactic acid by a species of *Rhizopus* can also be interpreted in terms of the various nutritional and environmental factors.

Molliard (*19, 20, 21*) has shown that, in the presence of all the necessary nutrient salts, *Aspergillus niger* develops an extensive mycelium without allowing the accumulation of acids; acids accumulate only when certain essential nutritive elements are lacking. Butkewitsch (*2*) suggested that the specific formation of acids by *A. niger* depends on the reaction of the medium. Kuznetsov (*9, 10*) attempted to establish a correlation between acid formation and the oxidation-reduction potential of the medium. Kanel (*7*) suggested that the formation of lactic acid by Mucorales, but not by other fungi, is due to the ability of the Mucorales to grow immersed in the liquid medium. He found that a change in the biochemical activities of the organism or its physiological polymorphism could be correlated with the specific nature of the organism and the oxidation-reduction potential of the medium. Kanel further attempted to differentiate between the fermentative and the oxidative phases of the fungus. The fact that aeration of the culture did not change the lactic acid yield led him to suggest that air acts only mechanically by mixing the liquid,

and since different oxidation-reduction potentials had no effect on the lactic acid yield, he believed that immersed *Rhizopus* growth is independent of the oxidation potential of the medium. A low potential repressed the formation of fumaric acid, however, which he considered to be a secondary oxidation product.

Plant physiologists (*24*) have long recognized two distinct phases in the process of energy utilization by cells of higher plants, namely, the fermentation phase, which comprises the production of lactic acid or of alcohol and CO_2, and the respiration phase, which results in the production of CO_2 and water. In the case of micro-organisms, lactic acid has usually been considered the final product of the anaerobic or fermentation reactions of certain specific bacteria. In discussing the metabolism of lactic acid bacteria, Kluyver (*8*) remarks that it is possible to have oxygen take part in the process; he adds, however, that "if respiration is defined as an oxygen consuming process which yields the energy necessary for the maintenance and proliferation of cells then the conclusion must be that lactic acid bacteria do not respire."

According to Neuberg (*22*), methylglyoxal forms the intermediary stage in the break-down of the sugar molecule to lactic acid. Meyerhof and Kiessling (*16*, *17*, *18*) and Embden et al. (*5*) do not ascribe to methylglyoxal any part in the process; they believe that phosphoglyceric and pyruvic acids form the important intermediary products. Lundsgaard (*12*, *13*, *14*) noted that the presence of sodium-monoiodoacetate, in certain concentrations, inhibits fermentation by yeast or by zymase, but has no effect upon oxygen consumption. He concluded therefore that sugar break-down in fermentations has no relation to its break-down in respiration. Some investigators believe that iodoacetic acid acts on the initial stages of glucose fermentation by inhibiting the phosphorylating mechanism, for in yeast (unlike muscle) no glucose disappears and no intermediate products are formed in the presence of iodoacetate (*4*).

In the experiments reported here, both lactic acid production and sugar break-down were repressed by iodoacetic acid, from which it may be assumed that lactic acid is a direct product of glycolysis. Other facts presented in this paper substantiate this assumption. The fact that a lower yield of lactic acid was obtained per unit of glucose consumed, both during growth and as a result of pellicle action, indicates direct oxidation of the glucose or of some of the precursors of lactic acid, even in the presence of the poison.

The following reactions are suggested to explain the mechanism of sugar break-down and respiratory process of *Rhizopus:*

$$C_6H_{12}O_6 \longrightarrow \underset{\text{(intermediate compound)}}{C_3H_6O_3} + \underset{\text{(intermediate compound)}}{C_3H_6O_3} \qquad \text{(anaerobic)}$$

$$\underset{\text{(intermediate compound)}}{C_3H_6O_3} \longrightarrow \underset{\text{(lactic acid)}}{C_3H_6O_3} \qquad \text{(anaerobic)}$$

$$C_3H_6O_3 \longrightarrow C_2H_5OH + CO_2 \quad \text{(anaerobic)}$$
(intermediate compound)

$$C_2H_5OH \xrightarrow{+\ 3O_2} 2CO_2 + 3H_2O \quad \text{(aerobic)}$$

$$C_3H_6O_3 \xrightarrow{+\ 3O_2} 3CO_2 + 3H_2O \quad \text{(aerobic)}$$

There are cases on record where lactic acid has been found to be an intermediary product in the metabolism of micro-organisms. Peterson and Fred (*25*) for example, have shown, that certain bacteria produce acetic acid through the lactic acid stage, by an oxidation process. The possibility that fumaric acid may be formed through this stage has also been indicated (*11*). However, the fungus under study could not initiate growth with lactic acid as the only source of energy.

The function of zinc in the metabolism of *Rhizopus* is of special interest. It has been recognized since the earliest works on the nutrition of fungi that these organisms require zinc, and this finding was recently confirmed for *Aspergillus niger* by Steinberg (*28*), Roberg (*26*), and others. However, considerable disagreement still exists concerning the role of this element in the metabolism of fungi. Some investigators have been satisfied to relegate the specific function of this element to that of a stimulant or biocatalyzer, whereas others have insisted that it is a normal nutrient, required in small amounts for the metabolism of the organism. Very little attention has been paid, in most of these investigations, to the role of zinc in the mechanism of transformation of the carbohydrate used by the organism as a source of energy. Oño (*23*) suggested that *Aspergillus* utilizes the carbohydrate more economically in the presence of $ZnSO_4$, the intermediary product, oxalic acid being oxidized more rapidly. Similar results were obtained by Butkewitsch and Orlow, (*3*) who found that zinc repressed acid formation, oxalic acid disappearing almost completely in its presence. Javillier (*6*) concluded that the role of zinc in the growth of *A. niger* is that of a catalyst rather than of a nutrient; this action was specific and could not be replaced by any other element. Wassiljew (*30*) suggested that zinc influences primarily the metabolism of the fungus, notably its acid production; that it is an essential element for growth and not a stimulant; and that both vegetative growth of the fungus and the formation and consumption of the acids from sugar are influenced by the presence of this element. The influence of zinc differs, however, with different strains of *A. niger*.

According to Roberg (*26*), zinc is essential to the growth of Aspergilli; however, in solutions that do not contain sufficient iron, it produces an injurious effect. An antagonistic relation was believed to exist between the two elements. A similar relation was observed by McHargue and Calfee (*15*) in studies of the effect of copper, manganese, and zinc on growth of fungi. Buromskiĭ (*1*) emphasized recently that zinc must still be looked upon as a stimulant for the growth of fungi; he went so far as to claim that even potassium and phosphorus should be placed in this category. Zinc was also found to increase the respiration coefficient of fungi, that is, the ratio of CO_2

to growth, and to modify the nature of the acids produced (*19, 20, 21, 30*). In the case of *Aspergillus niger*, this was correlated with a delay in spore formation resulting in increased dry weight and extension of the cycle of growth of the organism.

In the present studies, zinc was shown to stimulate both growth and spore formation of *Rhizopus*, but not to favor acid production. These results indicate that zinc either catalyzes the oxidation of glucose or of the precursor of lactic acid directly to CO_2, thereby increasing the liberation of energy and stimulating growth. One need not argue, at this time, whether the elements zinc and iron make conditions for the growth of *Rhizopus* "normal" or "abnormal." The very fact that the fungus was grown on an artificial substrate with a relatively high concentration of carbohydrate, with inorganic sources of nitrogen and minerals, makes conditions of growth sufficiently "abnormal," as compared with those under which the organism is commonly found in nature, that is, on stale bread or in soil. One is, therefore, hardly justified in concluding that because zinc stimulates the growth of the fungus, it makes conditions "normal," while iron, which alone is not favorable to growth but is favorable to lactic acid production, makes conditions "abnormal."

SUMMARY AND CONCLUSIONS

This paper presents the results of a study of the nutrition of a fungus belonging to the genus *Rhizopus*, its ability to produce lactic acid from different carbohydrates, the mechanism of production of the acid, and the role of this process in the metabolism of the fungus.

In general, conditions favoring the growth of *Rhizopus* were found to be unfavorable to the accumulation of lactic acid. The fully grown pellicle placed in a sugar solution produced lactic acid to the extent of 50 percent of the sugar under anerobic conditions and of about 60 percent under aerobic conditions; in the latter case some growth accompanied the process of acid formation. Only about 30 to 40 percent of the carbohydrate consumed was changed to acid in the absence of a neutralizing agent, in growth cultures, and as much as 70 to 75 percent in the presence of such an agent.

One may conclude from these results that *Rhizopus* produces lactic acid by a fermentation mechanism despite it being a strictly aerobic organism. The lactic acid is preceded by an intermediary substance, which is converted by pellicles, under anaerobic conditions, to the extent of 50 percent to the acid and the remaining 50 percent to alcohol and carbon dioxide. Only a little energy is liberated in this reaction. During growth of the organism, or under aerobic conditions, the precursors of lactic acid and of alcohol are oxidized partly to lactic acid and partly to carbon dioxide. The presence of different elements influences the predominance of one reaction over another. Zinc favors growth and is thereby unfavorable to lactic acid production; iron, on the other hand, is highly favorable to lactic acid production and is not favorable to cell growth.

The fungus *Rhizopus* is shown to possess both a fermentative and an oxidative mechanism, similar to those of higher forms of life.

The essential features of this paper were presented at the meetings of the Society of American Bacteriologists, December 27–29, 1937, Washington, D. C.

(Jour. Bact. *35*, 70, 1938). After this paper was accepted for publication, a discussion by Ward et al. (Ind. Eng. Chem. *30*, 1233–5, 1938) concerning certain phases of the intermediary metabolism and the role of ethyl alcohol in the respiration of a lactic acid producing *Rhizopus* appeared, in which the findings presented in this paper were confirmed.

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DETERMINATION AND OCCURRENCE OF BORON IN NATURAL PHOSPHATES, SUPERPHOSPHATES, AND DEFLUORINATED PHOSPHATE ROCKS[1]

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INTRODUCTION

Boron belongs to the gradually lengthening list of elements that are regarded as essential to plant growth. Recent papers review previous work on the effect of boron on plants (*1, 4, 8, 12, 26*)[3] and its occurrence in plants (*8*), soils (*8, 26*), and fertilizers including fertilizer materials (*5, 10, 11, 26*). The data for fertilizers and fertilizer materials, however, include only a few scattered results for boron in natural phosphates and superphosphates.

Results are given in this paper for boron in 54 representative samples of natural phosphates from various parts of the world, 9 samples of commercial superphosphates, and 3 samples of defluorinated phosphate rock. The results for boron in natural phosphates reported herein represent an extension of the studies of this Bureau on the composition of phosphate rock (*15, 18, 19*).

METHOD OF ANALYSIS

Chemical methods for determining boron have been classified and reviewed by Wilcox (*24*). The methods most commonly used for determining boron in minerals and fertilizers are the Chapin method (*23, p. 1691*), or some modification of it, and the official methods (*3, p. 32*). According to the Chapin method, boron is separated from the other constituents of the sample (fused with sodium carbonate, if insoluble in acid) by distillation with methyl alcohol in the presence of hydrochloric acid and of anhydrous calcium chloride as a drying agent. The methyl ester of boric acid formed in the absence of free water passes into the distillate, from which the boron is recovered in a small volume of water by saponification with alkali with subsequent removal of the alcohol by distillation. The alkaline aqueous solution of borate thus obtained is freed from carbonate and adjusted to a definite pH (6–7) after which the quantity of boron in the solution is determined by titration with standard alkali in the presence of mannitol to the pink color of the phenolphthalein indicator. In the official method for acid-soluble boron in fertilizers phosphoric acid, instead of hydrochloric acid, is used, and since phosphoric acid acts as a drying agent, this change renders the use of calcium chloride unnecessary.

The method used by the authors is substantially the method of Chapin, though the titration is in principle that of Foote (*9*). Diffi-

[1] Received for publication Sept. 14, 1938. Issued February 1939.
[2] The authors are indebted to K. D. Jacob, Fertilizer Research Division, who suggested the investigation and rendered valuable aid during the course of the work, and to J. I. Hoffman, National Bureau of Standards, for helpful suggestions on the application of the methods.
[3] Reference is made by number (italic) to Literature Cited, p. 914.

culties not met in the analysis of most materials heretofore studied are at once encountered in the application of the method to natural phosphates. Not only are the involved quantities of boron so small as to require reagents and apparatus that are sufficiently low in boron to reduce the blank correction to a very low value, but as a consequence of the presence of fluorine and silica in most natural phosphates the distillates contain relatively large quantities of hydrofluosilicic acid, which titrates in the same pH range (*17*) as does the complex of boric acid with mannitol. The authors' application of published methods to the determination of boron in fluorine-bearing phosphates and their observations on means of obviating the foregoing difficulties can be conveniently presented under the subjects: (1) Reagents, (2) titration of boric acid, (3) separation of boron from the sample, and (4) procedure used.

REAGENTS

Since the presence of fluorine in natural phosphates renders the glassware used in the course of their analysis a reagent in a very real sense, the use of borosilicate glassware is not permissible. Concentrated solutions of sodium hydroxide also become contaminated with boron from the glassware (*24*). Accordingly, Kavalier glassware (usually considered as boron-free) was used for the analytical operations and for the storage of alkaline reagent solutions. The boron content of different lots of the several reagent chemicals is shown in table 1. It may be pointed out that a 6 *M* solution of sodium hydroxide prepared from a more concentrated solution that remained in a Pyrex flask for several weeks contained far more boron (92 parts per million of B_2O_3) than did similar solutions prepared in Kavalier glass throughout (table 1).

TABLE 1.—*Boron in analytical reagents*

Reagent	B_2O_3 in reagent [1]		Reagent	B_2O_3 in reagent	
	As received	As used		As received	As used
	P. p. m.	*P. p. m.*		*P. p. m.*	*P. p. m.*
Sodium hydroxide	4	[2] 1	Calcium chloride, anhydrous for drying	53	2.4
Do	35	[2] 11	Methyl alcohol, absolute	4.5	0
Potassium hydroxide	7	[3]	Sodium carbonate	<5	<5
Do	66	[3]	Do	<5	<5
Calcium chloride, anhydrous for drying	32	.6	Mannitol	[4] 0	[4] 0

[1] Results include boron present in the hydrochloric acid used for neutralization.
[2] Milligrams of B_2O_3 per liter of 6 *M* solution.
[3] Not used.
[4] Result shown by blank titration on 2.5 g of mannitol.

A stock solution of carbonate-free sodium hydroxide (*13, p. 139*) was prepared by rinsing the sticks with distilled water to remove any surface boron arising from attack of the glass container, dissolving the rinsed sticks in an equal weight of distilled water, and allowing the solution to stand until the supernatant liquid was clear. Dilute solutions of carbonate-free sodium hydroxide were prepared as needed by diluting aliquots of this concentrated solution with recently boiled distilled water.

The reagent grades of anhydrous calcium chloride may carry appreciable quantities of boron (table 1). It was suggested [4] that the boron in this reagent probably comes from the glaze of the vessels in which it is dehydrated. Accordingly, boron from this source would appear for the most part on the surface of the granules, and its removal should be possible by extraction or volatilization. Nearly all the boron can be removed (table 1) in a single treatment by drenching 500 g of the salt in a liter copper beaker with 300 ml of 95-percent methyl alcohol containing 15 ml of concentrated hydrochloric acid and heating the well-stirred mixture on a hot plate and finally in an oven at 200° C. until the alcohol is expelled and the material again becomes anhydrous.

Absolute methyl alcohol for use in the isolation of boron from the sample was prepared by redistillation of absolute alcohol from a 3-liter Pyrex flask to which had been added a few sticks of potassium hydroxide. The presence of volatile organic acids in the alcohol would give high results for boron by the usual titration with two indicators (*13, p. 614*), and accordingly the 4.5 parts per million of B_2O_3 found in methyl alcohol as received (table 1) might be regarded merely as the B_2O_3 equivalent of the alkali consumed by such organic acids. However, the titration procedure used by the authors eliminates from the boron titration all acids that do not form complexes with mannitol, and, therefore, it would appear that boron was actually present in the alcohol. In any event the redistilled alcohol was very satisfactory.

As a matter of convenience a solution of mannitol, rather than the solid, was used in the titrations. The solution was prepared by dissolving 100 g of mannitol in recently boiled distilled water and making the volume up to 1 liter.

The sodium carbonate was used as received. Any boron in the hydrochloric acid would appear in the results for sodium hydroxide; however, the indications were that the acid contained considerably less boron than did the sodium hydroxide.

TITRATION OF BORIC ACID

In figure 1 are reproduced Foote's titration curves (*9*) for a buffered water (I) and the same containing added boric acid with (III) and without (II) mannitol. Accordingly, if the solution containing boric acid without mannitol is titrated (adjusted) until its pH value reaches some point *B* (curve II) in the pH range over which curves II and III are nearly parallel—7.6 in the present instance—a part of the boric acid will have been titrated, the alkali equivalent being $A'B'$. Then, on the addition of mannitol the pH value of the solution drops to B'' on curve III, and the titration can be completed by adding alkali (B' C' ml) until the pH value of the solution is again 7.6 at point *C*. The total alkali equivalent of boric acid is A' C', but since point A'' on curve II cannot, in general, be determined because of the limitations of indicators, as well as the presence of other acids that titrate in this pH range, the total equivalent has no meaning in actual practice.

[4] WICHERS, E. Private communication. National Bureau of Standards.

The problem, therefore, resides in the choice of the pH value to which the solution shall be adjusted before mannitol is added. For this purpose various indicators have been used; mention may be made of methyl red and paranitrophenol. With the former, the pH value lies at some point *E* on curve II; with the latter, at some point *D*. Since a different indicator, usually phenolphthalein, is used for the final titration, alkali is consumed in bringing a boron-free solution from the end point of one indicator to that of the other, which increases the blank correction to the titration. For example, Allen and Zies (*2*, *p.*

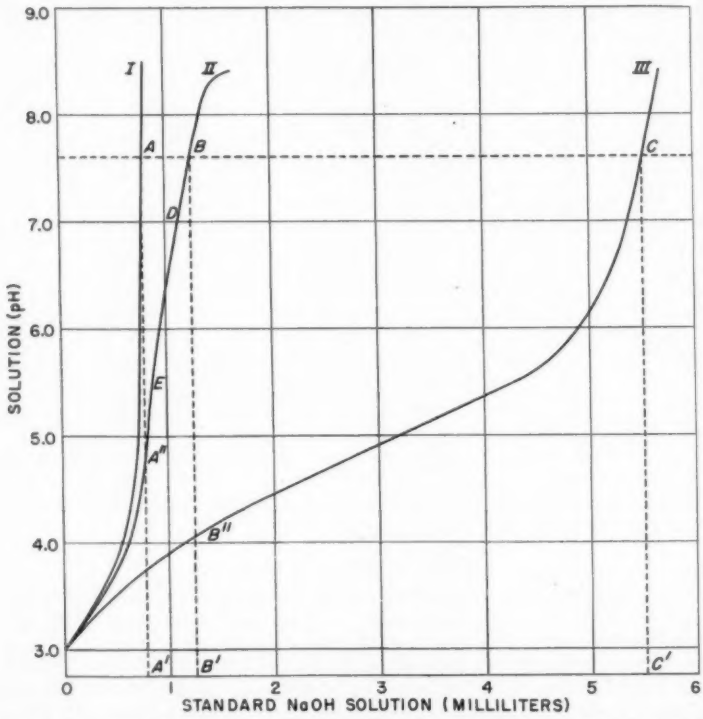

FIGURE 1.—Titration curves for boric acid (*9*). Curve I is for 50 ml of water, curve II for 50 ml of water plus 7.4 mg of B_2O_3, curve III for 50 ml of water plus 7.4 mg of B_2O_3 and 6 g of mannitol.

765) found the B_2O_3 equivalent of the alkali used in passing from the paranitrophenol end point to that of phenolphthalein to be 0.3 mg, which corresponds to a little less than 0.1 ml of 0.1 *M* sodium hydroxide, and the writers found the amount of alkali consumed between the methyl red and phenolphthalein end points to be 0.23 ml of 0.03 *M* sodium hydroxide. Furthermore, in the presence of weak acids or substances that titrate as weak acids, which are not readily separated from boric acid, as, for example, soluble fluosilicate, adjustment of the solution to a pH value in the approximate range *ED* with the necessary accuracy is extremely difficult, if not impossible. Moreover, the

titration of these same substances is continued after the addition of mannitol, thereby vitiating the result for boric acid.

The foregoing difficulties are obviated in Foote's method for the direct titration of boric acid in water (*9*) by adjusting the pH value of the solution before the addition of mannitol to the same value as that of the final end point. Since the two end points involve no pH change, other substances in solution do not interfere with the titration, except insofar as they render the end point difficult to establish. The small amount of boric acid neutralized during the initial adjustment ($A'B'$, fig. 1) is accounted for in the standardization factor of the alkali, and therefore the alkali equivalent used in practice is $B'C'$. Carbon dioxide, of course, must be excluded from the titration.

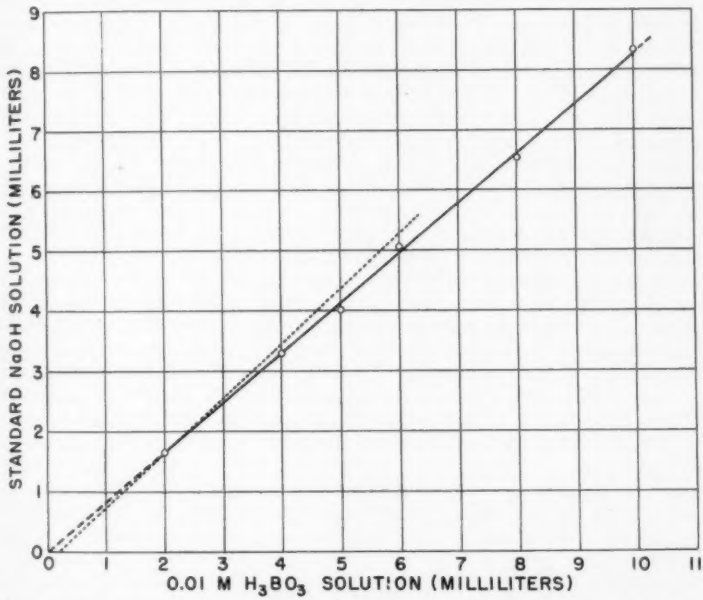

FIGURE 2.—Curve obtained in standardization of approximately 0.01 *M* sodium hydroxide against a 0.01 *M* boric acid solution with use of phenolphthalein as the indicator of both end points (10° C.). Volume of solution at initial end point was 100 ml, that at final end point was 125 ml plus the volume of standard sodium hydroxide added.

Foote adjusted his solutions to a pH of 7.6 colorimetrically with phenol red, and Wilcox (*25*) used an electrometric method. The authors used phenolphthalein, because of the comparative ease with which the end point can be recognized without accessory equipment. According to measurements with the glass electrode,[5] the faint pink of the phenolphthalein indicator in the concentration used by the authors appeared at the unusually low pH of about 7.6. In figure 2

[5] According to measurements made by L. M. White of the Fertilizer Research Division, the pH at which the faint pink color appeared in 100 ml of a 0.4 *M* solution of sodium chloride containing 20 drops of a 2-percent phenolphthalein solution was 7.6; with 10 drops of indicator the pH was 7.7, whereas with only 1 drop it was 8.4

are shown the results obtained in a typical standardization of sodium hydroxide solution against a boric acid solution prepared from fused pure boric acid (*13, p. 613*). The results fall on a straight line with a maximum deviation of about ± 0.1 ml of 0.01 *M* alkali. Since the curve passes through the origin, the blank correction is zero, as it should be with boron-free alkali, and the B_2O_3 equivalent of the alkali is obtainable directly from the slope of the curve. With an alkali containing an appreciable quantity of boron the curve would be rotated in the direction of the dotted line (fig. 2).

Besides carbon dioxide, which must always be excluded in the titration of boric acid, other known constituents of the analytical solutions obtained from natural phosphates that are likely to interfere in the subsequent titration of the boron are arsenic and fluorine. Allen and Zies (*2, p. 767*) found that the presence of 1 mg of As_2O_3 did not affect the titration of boric acid, but the presence of 25 mg rendered the phenolphthalein end point indistinct because of fading. The quantities of arsenic reported in natural phosphate (*15, p. 30*) are usually less than 0.005 percent of As_2O_3, which corresponds with 0.5 mg of As_2O_3 per 10 g of sample. Thus, if all the arsenic in the sample should follow the boron, the quantity present in the solution for titration would still be well within the tolerance indicated by Allen and Zies' results.

Quadrivalent germanium (*22*) and hexavalent tellurium (*20*) form complexes with mannitol that titrate as monobasic acids. Tellurium, like selenium, appears to be reduced to the metal in the presence of methyl alcohol, and thus it does not accompany boron in the alcohol distillate.[6] Although germanium behaves toward mannitol almost exactly as does boron, only a small fraction (probably 10 percent) of the amount of this element in the sample accompanies the boron in the final solution.[6] Thus, if the presence of germanium be neglected, the error thereby introduced in the result for boron (B_2O_3) would, because of the difference between the atomic weights of these elements, be equivalent to one-third of the germanium (GeO_2) accompanying the boron, or probably 3 to 4 percent of the germanium in the sample. Germanium has not been detected in phosphate rock, but, as far as the authors know, only two samples have been examined for this element (*14*).

The effect of fluoride and fluosilicate on the titration of boric acid apparently has not been studied to any extent heretofore. Allen and Zies (*2, p. 769*) noted some difficulty with the phenolphthalein end point in the titration of a solution obtained by distilling a synthetic mixture of boric acid and 0.2 g of NaF, whereas in a similar experiment with less fluorine (0.1 g of KF) Chapin (*23, p. 1691*) encountered no difficulty. The authors studied the effect of the presence of fluorine in quantities up to 100 mg by the addition of aliquots of a solution of sodium fluoride or hydrofluosilicic acid[7] to aliquots of standard boric acid solution and titration of the resultant solution for boron with phenolphthalein as previously indicated.

No interference was observed with sodium fluoride in the absence of notable quantities of free silica. On the other hand, with hydrofluosilicic acid pronounced fading of the indicator occurred at the

[6] HAGUE, JOHN L. Private communication. National Bureau of Standards.

[7] Prepared in the laboratory by adding an excess of pure quartz flour to c. p. hydrofluoric acid contained in a platinum dish, allowing the mixture to stand until the solution was clear, and decanting into a wax bottle.

final end point, and the result for boric acid tended to be decidedly low. This behavior is attributed to the re-formation of some fluosilicic acid as a consequence of the acidity developed in the boron-containing solution when mannitol is added to it. During the subsequent titration, the decomposition of the re-formed fluosilicic acid, which takes place slowly at the low temperature of the titration, would cause fading of the indicator and, unless the titration is continued until all the fluosilicic acid is destroyed, also a low titer for boric acid. The error arising from this source can be almost completely eliminated by continuing to add alkali until the pink of the indicator persists in the solution for at least 1 minute.

SEPARATION OF BORON FROM THE SAMPLE

The authors' recovery of added boron from synthetic mixtures and phosphate rock low in aluminum by distillation with phosphoric acid and hydrochloric acid is inclined to be 1 to 5 percent low as shown in table 2. Aluminum compounds show a marked tendency to prevent the distillation of boron, an observation also noted by others.[8] Early in the experiment, the use of phosphoric acid was discontinued because the results for acid-soluble boron obtained on phosphate rock with it were less than half the values obtained with hydrochloric acid. Later, however, it was discovered that if the rock-acid mixture is allowed to stand several hours before distillation, phosphoric acid alone gives as good results as hydrochloric acid and calcium chloride, provided only a very small quantity of water is present. For the distillation of samples that have been fused with sodium carbonate a larger quantity of phosphoric acid is needed to take care of the several milliliters of water added with the decomposed melt. The addition of phosphoric oxide (*6*) would seem preferable to the addition of larger quantities of acid.

TABLE 2.—*Recovery of boron by acid distillation*

Tabulation No.	Distillation mixture	B_2O_3			Fluorine present during titration
		Present	Found [1]	Recovered	
		Milligrams	*Milligrams*	*Percent*	*Milligrams*
1	10 ml 0.01 *M* H_3BO_3+10 ml concentrated H_3PO_4.	3.48	[2] 3.31–3.44	95.0–98.8	0
2	10 ml 0.03 *M* H_3BO_3+20 ml concentrated H_3PO_4.	10.44	10.13–10.31	97.0–98.8	0
3	1 ml 0.1 *M* H_3BO_3+20 ml concentrated HCl+20 g $CaCl_2$.	3.48	3.34	96.0	0
4	10 g phosphate rock No. 912+20 ml concentrated H_3PO_4.	----	[3] .34	----	147
5	10 g phosphate rock No. 912+20 ml concentrated HCl+20 g $CaCl_2$.	----	.33	----	67
6	Residue in distillation flask from 5+1 ml 0.1 *M* H_3BO_3.	3.48	3.52	100	3
7	10 g phosphate rock No. 912+1 ml 0.1 *M* H_3BO_3+20 ml concentrated HCl+20 g $CaCl_2$.	[4] 3.81	3.62	95.0	97
8	Same as 7+2 g Al_2O_3	[4] 3.81	3.13	82.2	2
9	10 g No.[5] 904+1 ml 0.1 *M* H_3BO_3+20 ml concentrated HCl+20 g $CaCl_2$.	3.48	2.90	83.3	0

[1] 400 ml of distillate were collected.
[2] The first 200 ml of distillate contained 3.20 to 3.34 mg of B_2O_3, and the second 200 ml 0.06 to 0.12 mg.
[3] Rock and acid were mixed and let stand overnight before distillation.
[4] Added B_2O_3 plus B_2O_3 found in 10 g of No. 912 (tabulation No. 5).
[5] Natural aluminum phosphate.

[8] ZIES, E. G. Private communication. Geophysical Laboratory, Carnegie Institution of Washington.

Relatively large quantities of fluorine and silica usually follow the boron. The observed quantities of fluorine were indeed variable and ranged from 2 to 147 mg (table 2), depending, among other things, on the quantity of acid used, the temperature in the distilling flask, and the composition of the sample. When 20 ml of hydrochloric acid were used in the distillation the quantity of fluorine following the boron usually ranged from 40 to 90 mg.

Since fluorine in the presence of silica has an adverse effect on the titration of boric acid, some simple means of separation is highly desirable. The addition of Al_2O_3 to the distilling flask is not permissible because it holds back the boron as well as the fluorine. Double distillation with hydrochloric acid reduced the amount of fluorine in the distillate about 50 percent, and double distillation in which only 3 to 5 ml of excess hydrochloric acid and 0.05 g of $ZrOCl_2$ were added to the flask before redistilling the second time lowered it still further to 10 mg or less without affecting the results for boron. The somewhat increased precision thereby obtained was not, in the author's opinion, sufficient to justify the additional time required for analysis.

Total boron, as distinguished from acid-soluble boron obtained by direct distillation of the phosphate sample, was determined in a few typical phosphate rocks by fusing the sample with twice its weight of sodium carbonate prior to distillation (*13, p. 614*). The results by the two methods of decomposition are shown in table 3. Obviously all the boron is not, in general, completely expelled from the sample by direct treatment with acid under the conditions that prevail in the distillation. The results by the two methods on Florida land pebble and two of the three Tennessee brown rocks are in substantial agreement, whereas in other instances (except No. 904) the results for total boron are considerably higher than those for acid-soluble boron. In the case of highly aluminiferous materials, such as soft phosphate (No. 580), the difference between the results is probably attributable to the retention of boron under certain conditions by aluminum compounds, as was noted in a preceding section. Available information relating to the boron-bearing constituents of phosphates does not justify any explanation for the disparity in the results for the other phosphates (table 3). It may be merely coincidental that the latter contain either relatively large quantities of organic matter (No. 1253), or sulphide minerals (Nos. 56 and 930), or both (No. 948).

TABLE 3.—*Boron in typical phosphate rocks as determined in fused and unfused samples*

Sample No.	Source or type of phosphate	Al_2O_3	B_2O_3 determined in sample—	
			Fused with Na_2CO_3	Unfused
		Percent	*P. p. m.*	*P. p. m.*
120 [1]	Florida land pebble	0.82	24	20
912	do	1.05	28	33
908	Tennessee brown rock	1.16	40	48
1253	Idaho rock	1.16	86	45
948	Wyoming rock	1.19	107	60
930	Tennessee blue rock	1.22	81	65
56a [1]	Tennessee brown rock	1.99	54	48
56 [1]	do	3.06	77	51
580	Florida soft	23.05	60	18
904 [2]	Grand Connetable Island	36.92	<10	<10

[1] Standard sample number of the National Bureau of Standards.
[2] Natural aluminum phosphate.

PROCEDURE USED

Add 20 ml of concentrated hydrochloric acid to 10 g of the sample in a liter flask of Kavalier glass. Swirl the flask until the contents are well mixed and then allow it to stand from ½ to 1 hour with occasional swirling. At the end of this period add 20 g of purified calcium chloride to the flask and mix by swirling; then add 50 ml of redistilled absolute methyl alcohol, and after mixing the contents by rapid swirling connect the reaction flask to the distilling apparatus (*13, p. 613*). Heat the reaction flask with a free flame until the contents begin to boil, then swirl the flask a few times without disconnecting it from the condenser, and replace the flame with a water bath maintained at 80° to 90° C. for the duration of the distillation. Keep the volume of alcohol in the reaction flask as constant as possible by distilling directly into it alcohol from a second distilling flask, which should be heated to boiling temperature before inserting the reaction flask to the system. Should the reaction flask run dry, the results will be low and erratic. Regulate the rate of distillation so that 400 ml of distillate will be obtained in 45 to 60 minutes.

Receive the distillate in a 500-ml Kavalier flask containing 30 ml of distilled water and 6 ml of 6 *M* sodium hydroxide solution, and stop the distillation when the contents of the receiver reach a volume of about 450 ml. When the distillation is finished add to the distillate 0.5 ml (10 drops) of a 2-percent solution of phenolphthalein and additional 6 *M* sodium hydroxide solution as needed to develop a permanent deep pink color.

If the sodium hydroxide carries an appreciable quantity of boron it will be necessary to measure the volume of alkali used, in order to make the proper blank correction to the result for boron. Accordingly, it is convenient to add the 6 *M* alkali in 6-ml portions. Ordinarily the alkali initially added to the receiving vessel is sufficient, though in runs on aliquots of a solution of boric acid alone and not infrequently with rock samples an additional 6 ml is required, and occasionally, when an unusually large quantity of fluorine distills over, as much as 18 ml of alkali will be needed.

After the addition of an excess of alkali to the distillate, distill off the alcohol by immersing the flask in a water bath with due care to avoid superheating at the beginning of distillation. Boiling tubes are indispensable here. When alcohol no longer distills over, transfer the pink residual aqueous solution directly to a 300-ml Kavalier flask for titration. It is not necessary to evaporate the solution to dryness and ignite the residue, as is done in the analysis of plant materials (*24*). Adjust the volume of the solution to 100 ml with water and boil the alkaline solution from 4 to 5 minutes on a hot plate to expel any residual alcohol before it is made acid incident to the removal of carbonate.

Render the hot alcohol-free solution acid with hydrochloric acid by adding concentrated acid until nearly all the excess alkali is neutralized, then 0.1 *M* acid to neutrality, and finally about 1 ml of 0.1 *M* acid in excess. Boil the acidified solution about 3 minutes with several vigorous swirlings during this period; make the solution faintly alkaline with 1 *M* alkali, then acid with 0.1 *M* hydrochloric acid, using 6 drops in excess, and reboil as before. Again restore the pink

color of the indicator with alkali, discharge it with 0.1 *M* acid, using this time only 2 drops in excess, boil again, and then cool the nearly neutral carbonate-free solution to 10° C. by placing the stoppered flask in a bath of ice water.

It is considered good practice to guard against a possible loss of boron during the boiling of the acidified solution by connecting the flask to an air condenser. The stepwise approach to the neutral point with intervening boiling not only increases the precision of the method but also lowers the blank titration on the reagents.

Adjust the cold (10° C.) milky solution accurately to the initial end point by adding 0.03 *M* sodium hydroxide solution to a permanent faint pink color, keeping the flask stoppered as far as possible to prevent the ingress of carbon dioxide, then add 25 ml of a 10 percent solution of mannitol. Boil the solution from 3 to 4 minutes with occasional swirling, again cool it to 10° C., and titrate it at this temperature with 0.03 *M* sodium hydroxide solution until the faint pink of the indicator persists for at least 1 minute. The result for boric oxide is obtained by multiplying the volume of sodium hydroxide solution (corrected for the blank titration on the reagents) consumed in the titration after the addition of mannitol by the factor for the alkali found by standardization against pure boric acid, the same titration procedure being used.

When only 6 ml of 6 *M* alkali was added to the alcohol distillate, the blank titration on the reagents amounted to 0.1 ml of 0.03 *M* sodium hydroxide, or 13 p. p. m. of B_2O_3 on the basis of 10 g of sample, which agrees well with the value (11 p. p. m.) expected from the analyses of the purified reagents (table 1). Furthermore, in the presence of the usual quantities of fluorine and silica the precision of the titration is also about 0.1 ml of 0.03 *M* sodium hydroxide. Accordingly, when the corrected titer was only 0.1 ml, the result is arbitrarily reported as $\leqq$10 p. p. m., whereas with a smaller titer the result is reported as $<$10 p. p. m.

Duplicate determinations of boron in the fluorine-bearing phosphates usually agreed within 15 p. p. m., the average difference being 8 p. p. m. Agreement was closer in the case of fluorine-free materials.

BORON IN NATURAL PHOSPHATES

Results for acid-soluble boron in domestic and foreign phosphates are given in tables 4 and 5, respectively. For the few results for total boron, as determined by fusing the sample with sodium carbonate, reference must be made to table 3. The data in tables 4 and 5 are summarized according to type of phosphate in table 6. South Carolina rock ranks first in quantity of boron and the African rocks second, each of these types of phosphate averaging more than 90 p. p. m. of B_2O_3. At the other extreme is bone ash, Florida soft and waste-pond phosphates, and Russian apatite, with less than 20 p. p. m. With the exception of the phosphatic limestone the averages for all other types of phosphate examined range from 42 to 61 p. p. m. of B_2O_3, inclusive.

TABLE 4.—*Acid-soluble boron in domestic phosphates*

Sample No.	Type of phosphate	Location of deposit	P_2O_5	B_2O_3
			Percent	*P. p. m.*
FLORIDA PHOSPHATES				
618	Land pebble	Pierce	30.53	98
619	do	Nichols	30.98	67
910	do	Mulberry	31.09	75
947	do	Brewster	31.28	72
790	do	Not known	31.40	70
912	do	Mulberry	35.37	33
120 [1]	do	do	35.40	20
1447 [2]	do	Bartow	35.11	54
1310 [3]	do	Not known	34.43	63
771	Hard rock	do	31.25	60
932	do	Dunnellon	35.99	40
580	Soft	Not known	29.49	18
728	do	Juliette	31.80	<10
581	Waste pond	Not known	18.18	<10
915	do	Dunnellon	23.63	<10
SOUTH CAROLINA PHOSPHATES				
495	Not known	Not known	16.07	63
1139	Land rock	Bulow mines, Johns Island	26.92	126
1138	do	Lamb's mine, near Charleston	27.85	142
1137	do	do	27.58	144
TENNESSEE PHOSPHATES				
56 [1]	Brown rock	Not known	31.28	51
56a [1]	do	Mount Pleasant	32.94	48
762	do	do	33.72	53
906	do	Wales	34.39	36
908	do	Mount Pleasant	34.44	48
1401 [3]	do	Wales	36.73	70
772	Blue rock	Glover	30.45	66
930	do	Gordonsburg	30.97	65
1049	Kidney	Boma	31.22	56
1048	White rock	Tomscreek	30.20	55
1031	do	Godwin	35.80	57
917	Phosphatic limestone	Gordonsburg	11.68	30
WESTERN PHOSPHATES				
550	Light colored	Idaho, Paris	32.21	67
1011	do	Montana, Garrison	27.63	32
1009	do	do	31.39	22
1252	do	do	36.38	48
1010	do	do	37.47	40
948	Dark colored	Wyoming, Cokeville	30.19	60
973	do	Idaho, Conda	32.53	70
1253	do	do	32.13	45
OTHER PHOSPHATES				
1295	Apatite	Virginia, Amherst County	39.58	47
971	Bone ash		40.36	17

[1] Standard sample number of the National Bureau of Standards.
[2] Concentrated by oiling and tabling.
[3] Concentrated by froth flotation.

TABLE 5.—*Acid-soluble boron in foreign phosphates*

AFRICAN PHOSPHATES

Sample No.	Location of deposit	P_2O_5	B_2O_3
		Percent	*P. p. m.*
1559	Algeria, Djebel Kouif	30.00	116
1549	Egypt, Safaga	32.79	110
593	Morocco	34.30	66
1551	Tunis, Gafsa	29.05	84

INSULAR PHOSPHATES

452	Christmas Island	39.46	46
985	Curaçao Island	38.59	70
904 [1]	Grand Connetable Island	54.51	<10
1230	Makatea Island	37.94	72
450	Nauru Island	38.92	55
451	Ocean Island	40.32	37

APATITES

905	Canada, Quebec Province	40.30	Nil
1305	Union of Soviet Socialist Republics, Kola Peninsula	31.63	12
1304	do	37.39	20

[1] Natural aluminum phosphate.

TABLE 6.—*Acid-soluble boron in different types of natural phosphates*

DOMESTIC PHOSPHATES

Type or source of phosphate	Samples analyzed	B_2O_3	
		Range	Average
	Number	*P. p. m.*	*P. p. m.*
Florida land pebble	9	20–98	61
Florida hard rock	2	40–60	50
Florida soft and waste pond	4	<10–18	<12
South Carolina	4	63–144	119
Tennessee brown rock	6	36–70	51
Tennessee blue and white rocks and kidney phosphate	5	55–66	60
Tennessee phosphatic limestone	1	----------	30
Western part of the United States:			
Light-colored rock	5	22–67	42
Dark-colored rock	3	45–70	58
Apatite from Virginia	1	----------	47
Bone ash	1	----------	17

FOREIGN PHOSPHATES

Africa	4	66–116	94
Islands	[1] 5	37–72	56
Apatite from the Union of Soviet Socialist Republics	2	12–20	16

[1] Excluding the sample (No. 904) of natural aluminum phosphate.

The authors' results for boron in phosphate rock are considerably lower than most of the figures reported in the literature. Young (*26, p. 32*), using the official method (*3, p. 32*) for acid-soluble boron, found 450 p. p. m. of B_2O_3 in a sample of Florida hard rock, only traces in two other Florida phosphates of unknown type, and 450 and 580 p. p. m. in two Tennessee brown rocks. The spectrochemical results of Gaddum and Rogers (*10*) are much higher. Thus, the latter authors report the equivalent of 0.16 to 1.6 percent of B_2O_3 in Florida pebble and soft phosphates. On the other hand, recent spectrochemical anlyses, made with extreme care by B. F. Scribner of the National Bureau of Standards (*14*), showed only 0.005 percent of B_2O_3 and <0.001 percent of B_2O_3, respectively, in a Tennessee brown rock (National Bureau of Standards standard sample No. 56a) and a

Florida land pebble (National Bureau of Standards standard sample No. 120). The results agree well with the authors' figures for these samples as shown in table 4.

At present, little can be said concerning the boron-bearing minerals occurring in natural phosphates. Schaller (*21, p. 168*) has prepared a list of all the known boron minerals including 56 names comprised in 2 groups, viz, 36 borates and 20 borosilicates. Many of these minerals are soluble in water and a large majority of them are either soluble in, or are decomposed by, hydrochloric acid. Several of the borosilicates, as, for example, tourmaline, are not decomposed by hydrochloric acid, though in the analysis of phosphate rock their decomposition would be promoted by the fluorine present in the sample. So far as the authors are aware, only one boron mineral has been identified in phosphate rock. A very small quantity of tourmaline was found (*15, p. 76*) in one sample of Florida land pebble (No. 912).

BORON IN SUPERPHOSPHATES AND DEFLUORINATED PHOSPHATE ROCK

In view of the presence of considerable quantities of fluorine in most natural phosphates and the volatility of boron fluoride, it would be difficult to predict the fate of the boron in phosphate rock during the manufacture and storage of superphosphate. In general, one might expect the boron to distribute itself between the superphosphate and the gases expelled when the rock and acid are mixed and during subsequent storage of the superphosphate. On the other hand, if boron-free acid were used and no boron were volatilized during manufacture, the boron content of the resulting superphosphate would, in the case of ordinary superphosphate, be roughly 50 percent of that of the ingredient rock. Thus, on the basis of the data in table 6, ordinary superphosphates prepared from Florida land pebble and Tennessee brown rock, respectively, would probably contain 10 to 50 and 18 to 35 p. p. m. of B_2O_3. These figures agree reasonably well with the amounts found by analysis (table 7).

TABLE 7.—*Acid-soluble boron in superphosphates and defluorinated phosphate rocks*

SUPERPHOSPHATES

Sample No.	Phosphatic fertilizer		P_2O_5	B_2O_3
	Type	Made from—		
			Percent	*P. p. m.*
1403[1]	Ordinary superphosphate	Florida land pebble	20.56	<10
1402[2]	do	do	20.60	<10
1580	do	do	20.85	51
1581	do	Tennessee brown rock	20.24	36
1486	Special superphosphate[3]	Florida land pebble	32.67	58
1361	Double superphosphate	do	49.27	94
1481	do	Tennessee brown rock	43.43	77
1362	do	do	48.37	158
1372	do	Idaho rock	47.33	96

DEFLUORINATED PHOSPHATE ROCKS

1476[4]	Fused phosphate rock	Tennessee brown rock	28.95	21
1351	Calcined phosphate rock	do	36.58	20
1478	do	do	37.25	30

[1] The sulphuric acid used in the preparation of this superphosphate was processed sludge acid from the raffination of asphalt-base petroleum.
[2] The sulphuric acid used in the preparation of this superphosphate was untreated sludge acid from the raffination of asphalt-base petroleum.
[3] Prepared by granulating a mixture of ordinary and double superphosphate.
[4] Defluorinated in the fused condition (7).

The agreement of the results in the foregoing comparison relating to ordinary superphosphate leaves the inference that in the manufacture of phosphoric acid by the wet process a large part of the boron in the phosphate rock may go into the product acid. Accordingly, double superphosphate made with such phosphoric acid would be expected to contain as much, or more, boron than the ingredient rock. The results for boron in double superphosphates (table 7), in comparison with the boron content of the same types of phosphate rock (table 6), clearly support this expectation.

Results for boron in domestic superphosphates reported by previous workers (*10, 26*) are considerably higher than the figures obtained by the authors, the differences being about the same as were found in the case of phosphate rock.

Without asserting that previously reported results are subject to such error, it should be pointed out in this connection that superphosphate, because of the presence of active fluorine compounds, readily attacks glass and cannot be stored in glass bottles without becoming contaminated with boron from the container. For the sake of comparison, results on portions of double superphosphates No. 1361 and 1362 that had stood in glass bottles for 3 years may be cited. Samples taken from the center of these bottles, that is, out of direct contact with the glass, showed 120 and 186 p. p. m. of B_2O_3, respectively, as compared with 94 and 158 p. p. m. (table 7) in material that was stored in wooden containers.

The quantity of acid-soluble boron found in defluorinated Tennessee brown-rock phosphate (*7, 16*) amounted to 20 to 30 p. p. m. of B_2O_3 (table 7), and since this material is almost completely decomposed by hydrochloric acid, these figures may be regarded as a close approach to the total boron. On the basis of the range of the results for brown rock (table 6) it appears that around 50 percent of the boron in phosphate rock is volatilized in the defluorination process. Defluorinated phosphate rock probably carries less boron than basic slag. An imported slag (not shown in the tables), the only slag examined by the authors, contained 116 p. p. m. of B_2O_3.

SUMMARY

The Chapin method, modified to the extent that only one indicator is required in the titration, was applied to the determination of the relatively small quantities of boron occurring in natural phosphates and superphosphates.

Results are given for boron in 54 samples of natural phosphates from various deposits of the world, 9 samples of superphosphates, and 3 samples of defluorinated phosphate rock. The results for acid-soluble boron (B_2O_3) in natural phosphates range from <10 to 144 p. p. m., in superphosphates from <10 to 158 p. p. m. and in defluorinated phosphate rock from 20 to 30 p. p. m.

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THE ANATOMICAL DEVELOPMENT OF LEPIDIUM DRABA[1]

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INTRODUCTION

The difficulties of weed control suggest the need for a fuller knowledge of the life histories of these plants. One of the most persistent perennial weeds in the West is hoary cress (*Lepidium draba* L.). In an effort to determine the cause of its resistance to eradication, a study was made of its anatomical development from the seed to the mature plant. The results are presented in this report.

MATERIALS AND METHODS

The studies of the anatomy of the seedling were made with plants grown from seeds secured from the Colorado seed laboratory and from seeds obtained from mature plants in the field. The mature plants used for the anatomical studies and those used as a source of seed were collected along a road and in a nearby field north of Loveland Lake, Loveland, Colo.

The studies on seedling development were made on plants collected weekly up to 6 weeks of age, then at 2 months, 3 months, 4½, and 5½ months. The plants 3 and 5½ months old were grown both in a garden plot and in the greenhouse. Plants of corresponding ages from the greenhouse and outdoor plots showed very few outstanding differences.

The paraffin method was used in most of the work, although free-hand sections were studied to check certain structures. Most seedlings were killed and fixed in chromo-acetic-formalin (42 cc of 1-percent chromic acid, 3½ cc of glacial acetic acid, 15½ cc of formaldehyde, and 39 cc of water). Alcohol-formalin-acetic fluid (48 cc of alcohol, 40 cc of water, 7 cc of formaldehyde, and 5 cc of glacial acetic acid) was used for the mature plant tissues. Because of the abundance of stone cells in the root and crown, and the large amount of lignified tissues in the older organs, it was necessary to treat these materials in hydrofluoric acid for 1 week before they were processed for sectioning in paraffin. Safranin-fast green was the stain combination used most frequently; safranin-Mayer's haem-alum was also employed.

EXTERNAL MORPHOLOGY OF THE SEEDLING

The cotyledons appear above ground 5 or 6 days after planting. The cotyledons expand in about 3 weeks to maximum size, approximately five times their dimensions in the seed. During this interval the first leaves emerge and begin to enlarge (fig. 1, 1–5). The leaves continue to enlarge for 5 or 6 weeks and form a loose rosette.

[1] Received for publication March 14, 1938; issued February 1938. Contribution from the Department of Botany, Colorado Agricultural Experiment Station.

[2] The author wishes to express his appreciation to Dr. L. W. Durrell, head of the Botany Department of Colorado State College, for consultation and for advice pertaining to these investigations, and to Dr. J. E. Sass and Dr. J. N. Martin of Iowa State College for help and suggestions in the preparation of the manuscript.

The radicle develops into a prominent taproot, from which lateral roots arise in 2 to 3 weeks. An enlarged region at the base of the hypocotyl, referred to as the "collet," marks the junction of the root and hypocotyl.

FIGURE 1.—Seedlings of *Lepidium draba* ranging in age from 1 to 13 weeks from time of planting seed, as indicated by numerals; *b* shows stem shoots arising from the hypocotyl.

Lateral stem shoots begin to appear in about 13 or 14 weeks, arising very often from the upper regions of the hypocotyl (fig. 2) and from the cotyledonary axils of the older plants. After this stage the plant begins to assume more mature vegetative characteristics. A descrip-

tion of the mature plant will not be given here, since it has been adequately described by others.[3]

ANATOMY OF THE EMBRYO

Preliminary to the embryonic studies, the seeds were soaked for a time in order to remove the seed coats, and the extracted embryos were embedded and sectioned in paraffin. Transverse and longitudinal sections (pl. 1, *A* and *B*) show that the cotyledons are reflexed nearly parallel to the radicle. The tip of the radicle has a thin root cap formed from a calyptrogen. The apical meristem of the epicotyl, between the curved cotyledons, consists of a small, conical mass of

FIGURE 2.—Seedlings of *Lepidium draba* showing secondary shoots arising from the hypocotyl region.

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cells. Some cells of the embryo appear to have two nuclei, and most of the cells contain an abundance of starch.

The central provascular cylinder of the radicle and hypocotyl branches just below the cotyledonary node and provides a branch for each of the cotyledons (pl. 1, *B*). The internal (ventral) surface of the cotyledons contains cells which form a palisadelike layer. The cells of the dorsal region are also very compact but more irregularly arranged.

THE ROOT

THE PRIMARY ROOT

The primary vascular system in the young seedling is a diarch protostele of the exarch type (pl. 1, *C*). In plants 1 week old, only the protoxylem areas are lignified. At this age the metaxylem cells are of mature size but are not lignified. Two small protophloem areas are present but are distinguished with difficulty from the pericycle. In plants at least 2 weeks old the phloem cells are usually smaller than those of the pericycle, and some sieve tubes and companion cells are recognizable.

The pericycle consists of one layer of parenchymatous cells. A supplementary layer of interstitial parenchyma fills in the area between the protoxylem and protophloem. The cells of the endodermis in the root of the 10-day-old plants are conspicuously larger than the pericyclic cells and may be recognized by the narrow elliptical casparian strips (pl. 2, *B*). Casparian strips were observed only in the lower part of the hypocotyl. The endodermal cells are thin-walled, very similar to the cortical cells although not quite so large in diameter.

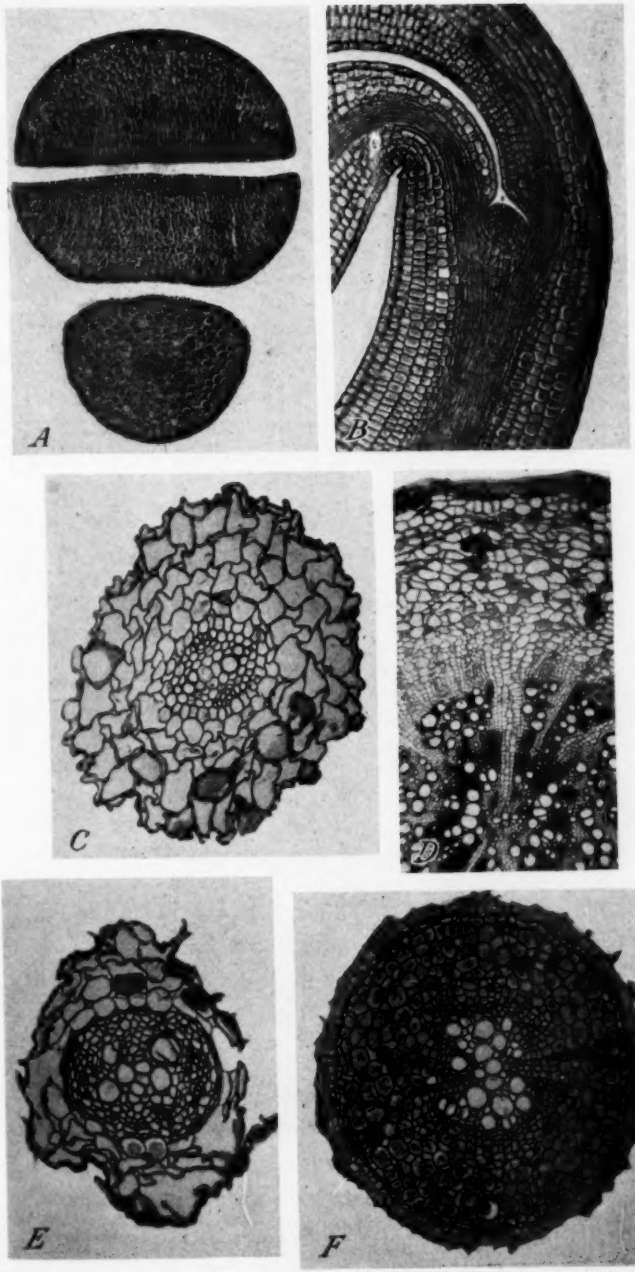
The wide cortex of the primary root consists of parenchymatous cells with large intercellular spaces. The very loose arrangement of the cells is conducive to the subsequent rapid break-down of the cortex. The epidermal cells are thin-walled and parenchymatous, varying in cross-sectional diameter both radially and tangentially In the region of absorption, root hairs are formed but are of short duration because of the early sloughing of the cortex.

DEVELOPMENT OF THE ROOT

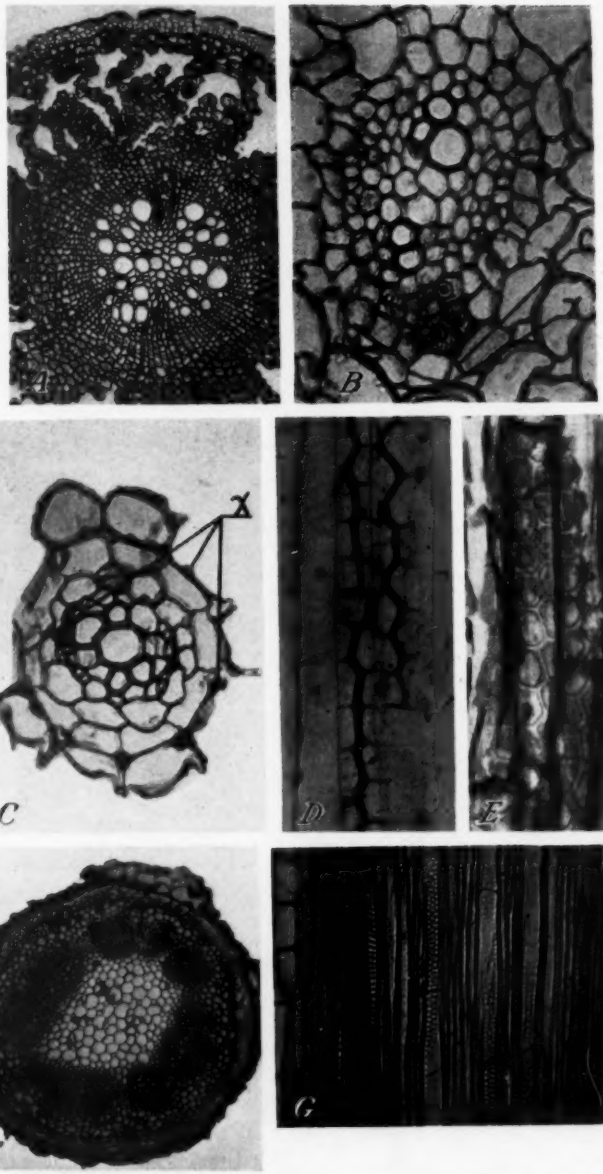
Structure of the Xylem

The formation of secondary xylem is initiated by a cambium between the small masses of primary phloem and xylem. The first secondary xylem cells are formed at right angles to and nearest the center of the diarch primary xylem (pl. 1, *C*, *D*). As development proceeds, fusiform cambial cells originate in the parenchymatous tissue tangential to those already present, gradually filling out the central, somewhat circular, area of xylem. After the first-formed masses of secondary xylem are as long as or slightly longer than the protoxylem areas on a radial plane, the cambium connects around the two points of primary xylem.

The lignified tracheae are interspersed among the parenchymatous cells and fiber tracheids. Extending radially from the protoxylem areas, wide parenchymatous xylem rays are laid down by the cambium. Narrow xylem rays also occur in the lignified areas of the xylem. The origin of these later-formed rays apparently is at the beginning of a new season of growth (pl. 1, *D*).



A, Cross section of seed embryo. × 40. *B*, Longitudinal section of seed through apical meristem. × 77. *C*, Section of week-old root. × 147. *D*, Section of 2-year-old root from field. × 31. *E*, Section of 4-week-old root. × 136. *F*, Section from 12-week-old root taken about 10 inches below ground line from green house flat. × 124.



A, Section about 2 inches below ground line of a 90-day-old root from garden plot. × 65. *B*, Section of 10-day-old seedling root. × 394. *C*, Section of small lateral root from 18½-week-old plant showing the inner cortical reticulum in cross section. × 485. *D*, Lignified corticular reticulum in innermost cortical layer from 18½-week-old primary root. × 418. *E*, Lignified corticular reticulum in innermost cortical layer from 12-week-old primary root. × 423. *F*, Section through one of the lower internodes of 18½-week-old stem. × 38. *G*, Longitudinal section of portion of vascular area in a mature stem. × 134.

Longitudinal sections of young and old roots show that the first protoxylem elements consist mostly of spiral tracheae, although some annular cells are formed. The secondary wall thickenings of these cells are very delicate. In the tracheae that are produced later, the secondary wall consists of pronounced scalariform thickenings. It was observed that a few elements formed at the end of the growing season were more like reticulate cells. The fiber tracheids have sharper pointed ends than typical tracheids, smaller lumina than the smallest tracheae, and greatly reduced bordered pits. The xylem parenchyma cells of the old roots are from two to five times as long as they are wide, and the majority of the cells have transverse or nearly transverse end walls.

Annual growth rings are formed in the portion of the root a few inches below the surface of the ground. The "springwood" contains relatively large tracheae and considerable parenchyma, whereas the "summerwood" contains smaller tracheae and a band of xylem fibers. Thus the difference in spring and summer growth makes the rings apparent.

Structure of the Phloem

The formation of secondary phloem is slow as compared with the rate of xylem formation. The phloem cells differ from the pericycle in that their walls appear a little thicker in cross section and stain slightly darker. The sieve tubes were found to have nearly transverse end walls, with the sieve plates located only at the ends. The angular companion cells are of the same length as the sieve tubes, very small in diameter, and have protoplasm slightly more dense than that of the sieve tubes.

The cambium produces a large amount of phloem parenchyma in which sieve tubes and companion cells are found in small isolated strands. However, stages were found where the phloem conducting tissue was difficult to recognize because of a wide radial band of undifferentiated cambial derivatives.

The older conducting phloem becomes crushed and is absorbed in the surrounding tissues as the plant axis continues to increase in diameter. This condition was observed in some sections from plants 12 weeks or more of age (pl. 1, *D*).

The Cortex

Very early in the life of the plant the cortex becomes wrinkled, breaks down, and sloughs away (pl. 1 *C*, *E*, and *F*). This breaking-down process is sometimes associated with the differentiation of a characteristic structure in the first layer of cortical cells next to the endodermis. A reticulate tertiary thickening is deposited on the inside of the inner tangential wall in these cells (pl. 2, *B*, *C*, *D*, and *E*). Not all young roots sectioned showed this structure. The youngest was 10 days old and the oldest was 18½ weeks old.

The thickenings stain red like lignified material when safranin and fast green are used as a staining combination. The meshes of the reticulum spread out flat in each cell and sometimes the open ends in two adjacent cells are opposite each other. This netlike structure was found only in the very young primary roots and in young secondary roots. It appeared to completely surround the stele of the

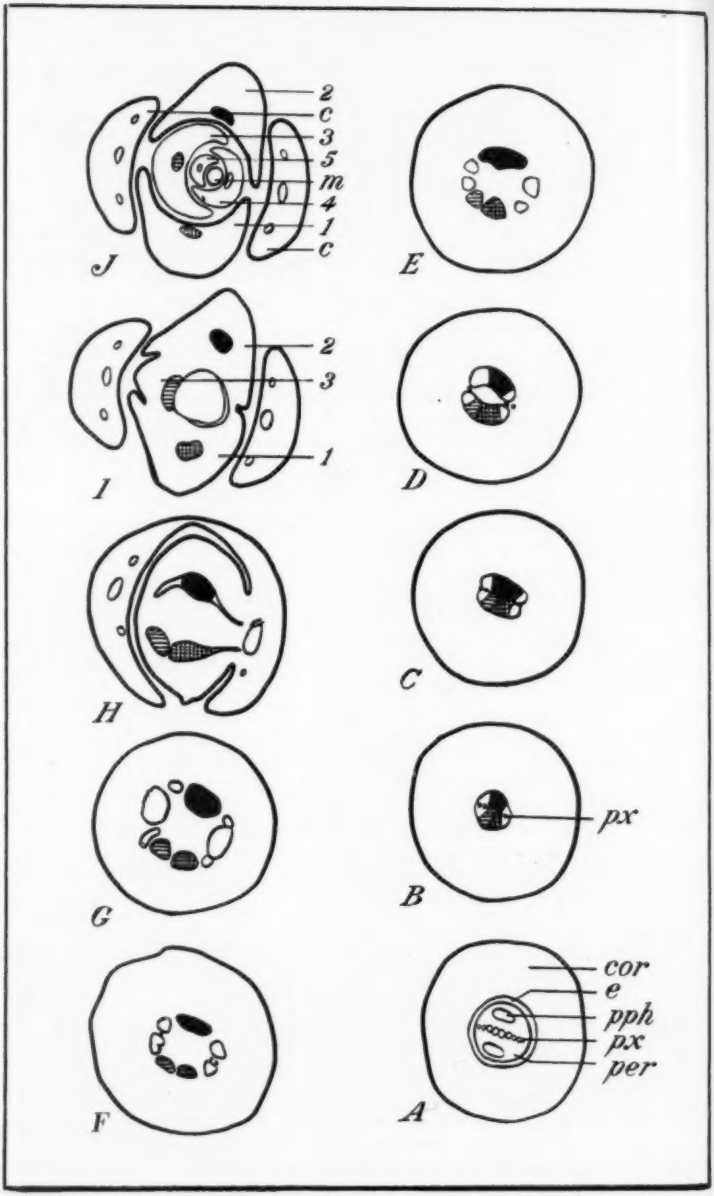


FIGURE 3.—*A–J*, Diagrammatic representation of a series of cross sections from a 3-weeks-old plant of *Lepidium draba* showing how each vascular trace of the young seedling arises from the stele below. In *I* the numbers 1, 2, and 3, and in *J* numbers 1 to 5 indicate the bases of the first three and the first five leaves, respectively. *cor*, cortex; *e*, endodermis; *pph*, primary phloem; *px*, primary xylem; *per*, pericycle; *c*, cotyledons; *m*, meristems.

roots of some young plants, while still younger roots had only a small patch on one side (pl. 2, *B*).

A periderm arises in the pericycle and is followed by the breaking down of the cortex. The phellogen produces a layer of cork which may attain a thickness of 12 or more layers of cells in old roots. However, the unbroken inner layer was only four or five cell layers in thickness. No evidence of the production of a phelloderm was found, all the tissue external to the cambium of the old root apparently being made up of secondary phloem cells.

Small clusters of these phloem cells in the "cortical region" of the root differentiate into stone cells as secondary phloem sclerenchyma. Plants taken from the garden plot October 10 showed stone-cell differentiation taking place, and most of the sections of old roots had stone cells in this tissue (pl. 1, *D*). The vascular tissues of the phloem are separated by phloem rays.

The 90-day-old root (pl. 2, *A*) had a considerable number of intercellular spaces in the "cortical region." These cavities extend vertically in the root for some distance. The exact length could not be determined from longitudinal sections because the cavities are not perfectly parallel with the axis.

Sections taken from roots 12 inches below the ground line did not contain as many of the intercellular spaces as sections from roots at 4- and 6-inch depths; while at 22 inches down, very small and few air passages occurred.

ROOT-STEM TRANSITION

The root-stem transition is most easily understood by studying seedling plants. The diarch protostele vascular arrangement of the root continues up into the hypocotyl to within a few millimeters of the cotyledonary node. The first indications of the transition change from root to stem are the production of slightly larger masses of secondary tissue situated at right angles to the line of the primary xylem, and the collapse of some of the primary xylem cells (fig. 3, *C*). A close examination of the center of a section, taken at a slightly higher level, shows the presence of two central parenchyma cells which is the first indication of the pith and of the siphonostele of the stem (fig. 3, *D*). Slightly larger masses of secondary tissue are also present and the primary xylem is reduced on each side to two tracheae.

A section taken from the hypocotyl about one-half millimeter higher has the secondary tissue divided into seven small areas separated by parenchyma cells. The secondary tissue contiguous to each side of the protoxylem points becomes separate from the semicircular masses, forming four bundles, represented as clear areas (Fig. 3, *E*). The secondary tissue on one side remains in one bundle and that on the opposite side separates into two smaller bundles. Figure 3, *F*, is similar to *E*, differing only in the fact that *F* shows the first indication of the separation of a small mass of vascular tissue to form a lateral cotyledonary trace. This can be seen as a partly divided bundle (clear) at the left side of figure 3, *F*.

No evidence of the primary vascular system can be found at the plane where the section for figure 3, *G*, was taken. The two small bundles at the sides of the primary xylem points fuse and send out small bundles which will become lateral bundles of the cotyledons.

The changes that take place at successive levels are evident in figure 3, *H*, *I*, and *J*. In *I* the central vascular ring shown in *H* has divided to become the vascular traces for the first three leaves. The section cut through the apical meristem (fig. 3, *J*) shows the cotyledonary bases at the outside extremities with the base of five leaves in between them and the meristematic tip in the center.

THE STEM

PRIMARY STEM STRUCTURES

Since the hypocotyl up to a few millimeters below the cotyledons is a diarch protostele like the root already described, a detailed description of the hypocotyl will not be necessary. The principal anatomical difference between the root and hypocotyl is that the cortical region of the hypocotyl is larger in diameter than that of the root and its epidermis contains stomata. The root-stem transition, which takes place a short distance below the cotyledons, has been described.

A prominent shoot is not evident until the plants are at least 12 weeks old. The protoxylem consists largely of spiral elements, although a few annular and scalariform protoxylem cells are formed. The metaxylem contains a few reticulate and pitted elements. The first-formed protophloem appears to be phloem parenchyma, although very early the sieve tubes and companion cells are in evidence in small restricted patches.

A cross section of an internode of an 18½-week-old stem discloses a large pith with large intercellular spaces and an irregular medullary sheath adjacent to the primary xylem (pl. 2, *F*). The vascular cylinder consists of vascular bundles, some of which are distinct while others are joined by vascular tissues formed by the interfascicular cambium.

The xylem is made up of small clusters of lignified tracheae interspersed among parenchymatous cells and partially differentiated fibers. The cambium cells are small, thin-walled, and somewhat rectangular in cross section. The phloem at this age consists of sieve tubes, clearly defined companion cells, and undifferentiated fiber cells.

In many of the plants grown in greenhouse flats and in some of the stem shoots of field plants the cortex of the stem near the ground had collapsed and disintegrated. This condition was found in plants growing in a crowded environment. Under such conditions, a periderm was found just outside the phloem, probably arising in the innermost part of the cortex. Thus the "cortical region" of such an organ (pl. 2, *F*) is largely made up of old phloem, limited on the outside by the thin periderm layer. The cortex higher up on the stem is not sloughed off but remains as a loosely arranged tissue with large intercellular spaces (pl. 3, *A*).

CHANGES IN THE MATURE STEM

The more mature stem structures can be seen in plate 3, *C*. Besides the noticeable increase in vascular tissue certain other changes are apparent. In the cortex of the stem the intercellular passages are larger and appear to be more numerous than in the younger stem. These passages are arranged in such a way as to give a netlike appearance to regions of the cortex. The epidermal cells of the stem appear

rectangular from external view and are nearly isodiametric in cross section. These thick-walled cells are cutinized on both the inner and outer tangential walls. A slightly thinner-walled hypodermis was evident on the mature stem (pl. 3, *C*). The guard cells of the stomata are small and slightly sunken. Their cross-sectional area was about one-fourth that of the normal epidermal cells in close proximity to them.

Epidermal hairs, uniform in shape, are commonly present over most of the aerial organs of the plant. Their structure will be described later as part of the leaf. The hairs on the subterranean portion of young stem shoots just emerging from the ground in moist soil are more numerous and longer but otherwise similar to root hairs (pl. 3, *B*).

These modified epidermal hairs were not equally abundant around the entire circumference of the stem. They were longer and thicker on the lower side of the bend where the shoots were attached to the old crown. Since these hairs were found early in the spring and were not present on older shoots later in the growing season, they are considered to be temporary structures. The indications are that under the environmental conditions encountered early in the spring (April 15), these modified stem hairs function as root hairs for a time, but as the top layer of the soil dries out and a periderm develops in the pericycle of these underground organs, cutting off the outer tissues, the cells dry up and disappear.

THE CROWN

During a severe winter the crown and even some of the top of the root may be killed. When this occurs, new crown shoots are formed as adventitious buds near the top of the living section of root. In the plots where the tops of the roots were dug for anatomical studies, from one to four new shoots were sent up through 12 inches of soil. The lower 9 or 10 inches of these shoots developed crown characteristics and the remaining portion had stem structure.

Since the early development of the crown was found to be similar to that of the aerial stem, the development of the two organs will not be discussed separately. Both young stem and crown have a ring of vascular bundles forming a dissected siphonostele, with rays of parenchyma connecting the pith with the cortex. In the crown cambial activity is more rapid than in the stem, resulting in the early formation of a continuous cylinder of thick-walled lignified xylem (pl. 4, *A*).

As in the root, the primary cortex of the crown is sloughed off early in the life of the organ. A phellogen arising in the tissues outside the primary phloem forms a protective layer of phellem. Since the endodermis could not be identified in the crown, the exact tissue giving rise to the phellogen was not determined. Judging from the amount of parenchyma cells inside the periderm, the phellogen in the crown probably arises in the primary cortex.

The crown of old plants develops clusters of stone cells in the parenchyma outside the active phloem early in April (pl. 4, *A*), whereas the seedling plants in deep greenhouse flats did not form the stone cells until August, when the plants were 6 months old. Intercellular spaces were formed in the outer portion of old phloem of the crown the first year in plants grown both in the greenhouse and in the garden plot.

Comparisons of old crowns with the lower part of mature stems did not reveal any striking structural differences. In the upper levels of the mature stem isolated masses of fibers occur just outside the active phloem (pl. 3, *C*). The pith of the stem is much larger in cross section

FIGURE 4.—*Lepidium draba*, showing new stem shoots arising from the crown shoots and also a portion of the root system.

than the pith of the crown. The stem contains tracheids, whereas the crown had thick-walled, long fiber tracheids with sharp tapered ends. The pitted tracheae of the crown and stem have large pit openings, which when greatly magnified show the torus to be perforated (pls. 3, *D* and 2, *G*).

ADVENTITIOUS SHOOT AND ROOT FORMATION

The root system of the plant has both perpendicular and horizontal roots (figs. 4 and 5). No structural differences were found between the horizontal and the perpendicular roots and both produce adventitious buds which develop into shoots. Under field conditions the horizontal roots send up the most shoots and thus are the principal means of vegetative spread of the plant. If the top of a vertical root is killed by winter freezing or is cut off in digging, new aerial shoots arise opposite a large xylem ray in the phloem. Plate 4, *C*, shows a shoot arising from an old root.

In the seedling stage, supplementary stem shoots commonly arise from the middle or lower portion of the seedling hypocotyl (fig. 2 and fig. 1, 13, *b*). Since that part of the hypocotyl was found to be a protostele similar to the root, the formation of these buds is similar to buds formed on the vertical roots.

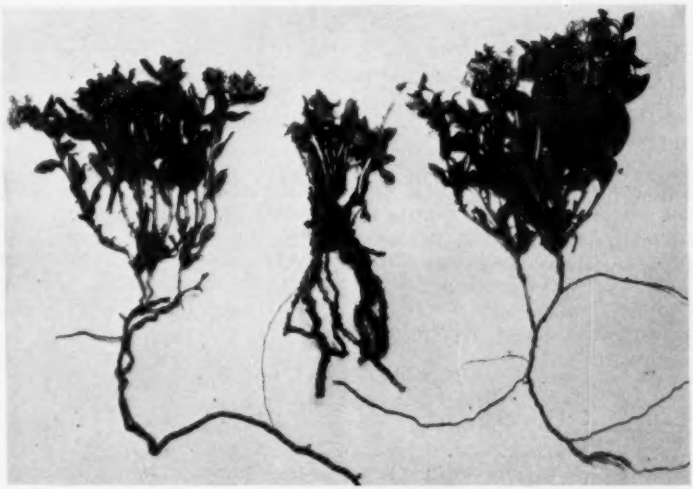


FIGURE 5.—*Lepidium draba:* Outside plants in bud and early blossom stage, with part of root system. Central plant from different plot slower in development; section from root showed five growth rings.

Adventitious roots are also formed from young stem shoots below the ground line. These roots originate from the pericyclic region radially between primary bundles of young stem shoots (pl. 4, *D*).

THE LEAF

Although the external appearance of the leaf has been adequately described by taxonomists, certain external structures should be mentioned here. The pubescence on the leaf surfaces consists of long pointed one-celled trichomes. The trichome cell has a thin covering of the cuticle over its surface. When greatly magnified the hairs appear to have numerous very small elongate stipulate pit markings in their walls. The trichome distribution over the leaf surface was

found to be fairly uniform on the various plants examined. Data taken from 10 leaves from each of 10 scattered plants gave an average of 30 hairs per square millimeter on the lower surface and 25 per square millimeter on the upper surface.

The average number of stomata on the upper and lower surfaces was found to be 273 per square millimeter or 173,355 per square inch on the upper surface of the leaf and 267 per square millimeter on the lower surface. These average numbers are not considered to show a significant difference.

A study of the internal structure of the leaf (pl. 4, *B*) shows it to be very compact. There is marked uniformity of structure in the mesophyll, and intercellular spaces are few. In most of the leaves the mesophyll consists of short palisadelike cells, but in some leaves a poorly developed spongy parenchyma layer was found. Plate 4, *B*, *s*, shows a cross section of a stoma. The transfusion cells around the vascular system of the leaf show best around the large vein bundles.

SUMMARY

The embryo of the dormant seed of hoary cress (*Lepidium draba*) consists of two reflexed cotyledons, a small dome-shaped apical shoot meristem between the cotyledons, and a cylindrical radicle. The provascular system is evident in the axis and cotyledons and consists of undifferentiated procambial cells.

The stele of the root is exarch and diarch.

The root-stem transition is only a short distance below the cotyledons. The protoxylem points are the last of the primary xylem to disappear, and flanking masses of xylem fuse to form the vascular supply to the coytledons. Primary plumular development is independent of that of the primary vascular system of the root and plumule elements are continuous with secondary tissues formed by the cambium in the region of the hypocotyl.

The cortex of the primary root is sloughed off very early; the "cortex" of the older root consists of old phloem.

A secondary wall thickening is deposited in the first layer of cortical cells next to the endodermis of some young primary and secondary roots.

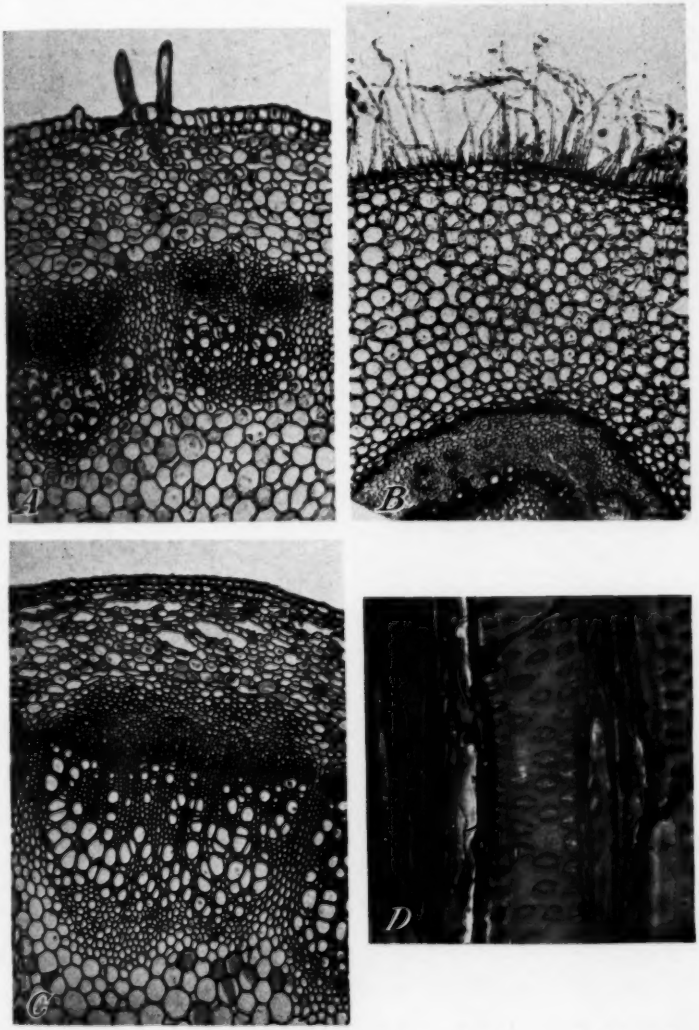
Old roots and the seedling hypocotyl produce adventitious shoots readily.

Less commonly stem shoots form root meristems below the surface of the ground.

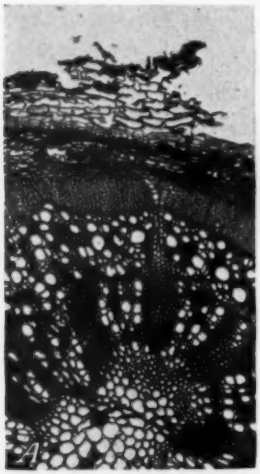
Modified stem hairs having characteristics of root hairs were found below the ground line on young stem shoots.

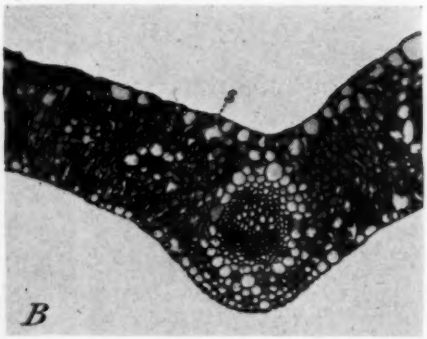
The leaf had a very compact structure suggestive of xerophytic plants and the root cortex of the first 8 inches below the ground contained large intercellular areas somewhat like those of hydrophytic plants.

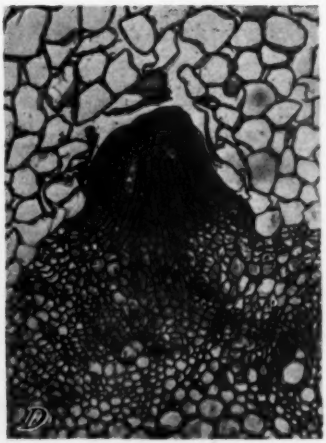
The leaf stomata are extremely small and very numerous, numbering as many as 173,355 to the square inch.



A, Section of young stem. × 96. *B*, Section from below ground of young stem shoot showing stem hairs modified as root hairs. × 43. *C*, Section of mature stem. × 75. *D*, Longitudinal section of pitted trachea showing perforated torus. × 708.







A, Section of crown showing the lignified rays. × 58. *B*, Section of mature leaf. × 67. *C*, Section through a root showing stem shoot arising from the root. × 46. *D*, Section through stem shoot showing the origin of an adventitious root. × 101

BLACK RING, A VIRUS DISEASE OF CABBAGE AND OTHER CRUCIFERS[1]

By C. M. TOMPKINS, *assistant plant pathologist*, M. W. GARDNER, *plant pathologist*, and H. REX THOMAS, formerly *graduate assistant in plant pathology, California Agricultural Experiment Station*[2]

INTRODUCTION

A virus disease of cabbage (*Brassica oleracea* L. var. *capitata* L.), designated as black ring, is prevalent mainly during the winter and spring seasons near San Pablo and Colma in the San Francisco Bay section and in the Sacramento, Salinas, and Santa Clara Valleys of California (*18*).[3] Other crucifers of more importance in California, including Brussels sprouts (*B. oleracea* L. var. *gemmifera* DC.) and cauliflower (*B. oleracea* L. var. *botrytis* L.) are also highly susceptible to infection. The results of field and greenhouse studies of this disease, in progress since 1932, are presented in this paper.

REVIEW OF LITERATURE

A mosaic disease of cabbage and other Brassicae, characterized by clearing and necrosis of the veins and yellowing and dwarfing of the plants was briefly recorded by Ogilvie et al. (*8*, *9*). Wallflower plants were also found to be susceptible to infection which induced breaking of the flowers.

In 1935, Smith (*12*) described a virus disease of cabbage, Brussels sprouts, and other species of *Brassica*, prevalent near Cambridge, England, which he designated as "ring spot." It seems probable that this disease was identical with that referred to by Ogilvie et al. (*8*, *9*). The principal symptoms on cabbage and Brussels sprouts leaves, especially the older ones, consisted of sunken, black, necrotic rings scattered uniformly over the surface. By means of juice inoculations, infection was obtained on cabbage, *Nicotiana glutinosa* L., *N. langsdorffii* Weinm., and *N. tabacum* L. var. White Burley. The insect vector proved to be the green peach aphid (*Myzus persicae* (Sulzer)). Later, Smith (*13*, *14*) reported natural infection of wallflower (*Cheiranthus cheiri* L.), annual stock (*Matthiola incana* R. Br. var. *annua* Voss), Brompton stock (*M. incana*), *Arabis* sp., and sweet rocket (*Hesperis matronalis* L.) with the virus of "cabbage mosaic." Apparently the name cabbage mosaic was used in a sense synonymous with ring spot, but this has led to some confusion. The designation of this disease as ring spot is considered unfortunate in view of the earlier use of this term by Weimer (*21*) to describe a ring spot of crucifers caused by *Mycosphaerella brassicicola* (Fr.) Lindau.

Hoggan and Johnson (*6*), in describing a virus disease of crucifers, observed a mild mottling or chlorotic spotting on the leaves of cabbage seedlings inoculated at temperatures of 70° to 80° F. This virus

[1] Received for publication May 18, 1938; issued February 1939.
[2] The writers are indebted to Prof. B. A. Madson and W. W. Mackie for greenhouse space and facilities. Valuable assistance in the greenhouse work was rendered by employees of the Federal Works Progress Administration.
[3] Italic numbers in parentheses refer to Literature Cited, p. 951.

was readily transmissible both mechanically and by means of the cabbage and green peach aphids. No mention was made, however, of necrotic rings as a symptom on this host.

Although the reference by Smith (*12*) is apparently the only one which may directly relate to the black ring disease of cabbage herein discussed, it should be noted for the sake of clarity that certain other crucifer viruses may also cause necrotic lesions on cabbage. These, however, are of a different type. Blank (*1*), workers at the Wisconsin station (*2, 3*), and Walker and Larson (*7, 20*), have discussed a mosaic disease of cabbage, prevalent in Wisconsin, which is characterized by mottling followed by the formation of small, necrotic spots on all of the head leaves. During a recent visit in California, R. H. Larson was shown typical specimens of the black ring disease of cabbage and concluded that it is different from the mosaic disease of cabbage in Wisconsin. Necrotic lesions were produced on cabbage seedlings inoculated with a turnip mosaic virus from New York in recent tests (*16, fig. 5*). These proved to be entirely different from those caused on cabbage plants by the cabbage black ring or the Wisconsin cabbage mosaic virus. Jones [4] reported a mosaic disease of cabbage in Washington but made no mention of necrotic lesions. Recently, through the courtesy of J. F. Adams of Mount Vernon, Wash., the writers received specimens of a virus disease on cabbage in Washington which is similar to, and may be identical with, the black ring virus disease in California. The host range of the Washington cabbage virus closely parallels that of the black ring virus, but the properties have not been studied.

Although Clayton (*4*) found that the rutabaga mosaic virus did not cause necrotic lesions to appear on cabbage leaves, either by natural or experimental means, it is of interest to note that this virus did induce yellow blotches surrounded by necrotic edges on Brussels sprouts leaves (*4, fig. 1, B*).

SYMPTOMS OF THE DISEASE

The symptoms of the disease produced on Winter Colma cabbage by mechanical inoculation in the greenhouse are identical with those observed on different field-grown varieties of cabbage exposed to natural infection. Early symptoms consist of numerous, small, chlorotic, circular to irregular lesions, ranging in size from 0.5 to 1.0 mm in diameter, which are distributed at random between and adjacent to the veins (fig. 1, *A*). When viewed in either reflected or transmitted light, the chlorotic lesions are more prominent and distinct on the upper than on the lower surface of the leaf. Within a few days after the lesions appear there may be a change in color to light viridine yellow [5] accompanied by an increase in size from 1 to 3 mm in diameter. Simultaneously, the chlorotic lesions become more abundant, and there is a gradual transition during which the normal, dark-green color of the leaf is replaced by the chlorotic areas. This gives a marked chlorotic appearance to leaves of infected plants (fig. 1, *B*). Small, dark-green islands now appear against a chlorotic background (fig. 1, *B*). Occasionally, in the early stages of infection, some leaves may show necrotic lesions, chiefly as very small, dark-olive specks and a few large chlorotic areas surrounded by broken to entire, necrotic edges, between and on

[4] JONES, LEON K. OBSERVATIONS ON PLANT DISEASES IN WASHINGTON IN 1936. U. S. Bur. Plant Indus. Plant Disease Reptr. 20: 230–235. 1936.

[5] Color determinations were made with the aid of Ridgway's (*11*) manual.

the veins (fig. 1, *C*). Two to three weeks after the initial appearance of the chlorotic lesions, a necrotic margin develops on many of them, in general less than 0.25 mm in width (fig. 1, *D*), in marked contrast to a normal, healthy cabbage leaf (fig. 1, *E*). When examined with a hand lens, these necrotic rings are seen to be surrounded by a narrow, light-green to light-yellow halo. Not all chlorotic lesions become necrotic. Eventually considerable coalescence of lesions occurs,

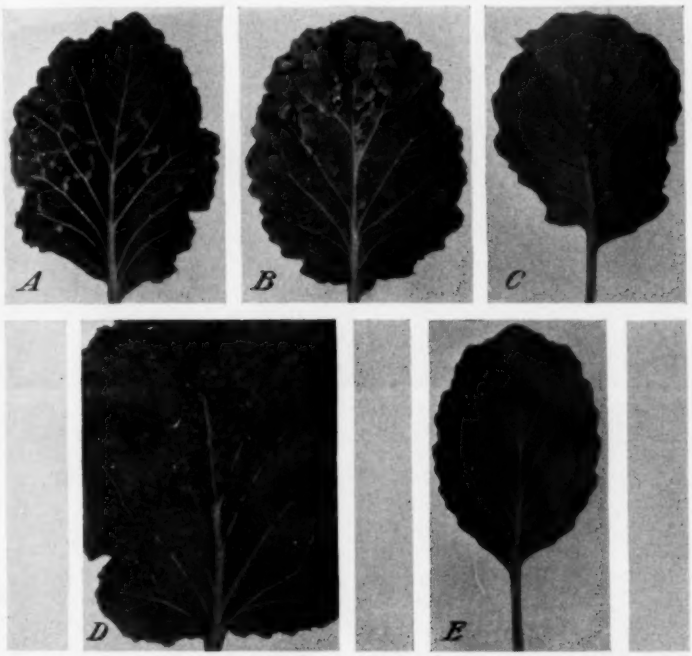


FIGURE 1.—Symptoms produced by the black ring virus on Winter Colma cabbage leaves by mechanical inoculation in the greenhouse at 13° to 19° C.: *A*, Chlorotic lesions on a normal, dark-green background; *B*, dark-green islands on a chlorotic background; *C*, necrotic specks and lesions; *D*, necrotic lesions in late stages of infection; *E*, noninoculated control.

resulting in an irregular, multiple lesion. Where lesions abut directly on a vein or on the midrib, the latter become blackened and shriveled. Eventually the chlorotic to yellow centers of many of the lesions surrounded by necrotic rings become entirely necrotic. The necrotic lesions are generally more numerous and more conspicuous on the lower leaf surface. As the older infected leaves commence to turn yellow with age, the lesions on the upper surface become somewhat more prominent; this is also true of the lesions on the lower surface.

Under natural conditions, symptoms are visible principally on the older, outer head leaves (fig. 2, *A*), although affected plants may be stunted in size. Early and late stages of these necrotic lesions on cabbage leaves, greatly magnified, are shown in figure 2, *D* and *E*. As with greenhouse-infected cabbage plants, the necrotic lesions are most

conspicuous on the under surface of the leaves. When viewed from a distance, a field of infected cabbage plants looks as though it had been scorched by fire. Similar symptoms occur on naturally infected cauliflower leaves (fig. 2, *B*) and on sprouting broccoli (*B. oleracea* var. *botrytis*) (fig. 2, *C*).

In the greenhouse, chlorotic lesions are sometimes larger and more conspicuous on cauliflower than on cabbage leaves (fig. 3, *A*), and in

FIGURE 2.—Necrotic lesions produced under natural conditions by the black ring virus: *A*, On cabbage; *B*, on cauliflower; *C* on sprouting broccoli; *D*, single or multiple lesions with green centers on cabbage; *E*, single or multiple lesions, on cabbage, showing complete necrosis.

marked contrast to a healthy cauliflower leaf (fig. 3, *B*). These vary in size and shape and, as with cabbage, some of them may change in color to light viridine yellow. Although necrotic lesions occur on the leaves of naturally infected cauliflower, attempts made in the greenhouse to reproduce this symptom with juice from naturally infected cabbage and cauliflower plants and from the stock culture of the black ring virus maintained on cabbage plants in the greenhouse have all been unsuccessful.

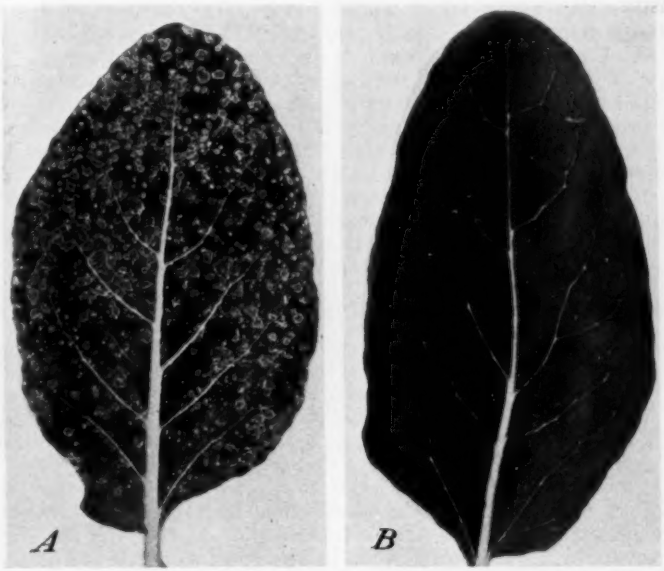


FIGURE 3.—Symptoms produced by the black ring virus by mechanical inoculation in the greenhouse at 13° to 19° C.: *A*, Chlorotic lesions on February cauliflower leaf; *B*, noninoculated control.

TRANSMISSION

The strain of the black ring virus used in studies on transmission, host range, and properties was derived from a diseased cabbage plant collected at San Pablo, Calif., in November 1935. The virus was established on young cabbage plants, variety Winter Colma, in the greenhouse and has been maintained by monthly transfer without any apparent change in virulence. Winter Colma cabbage seedlings were used as the standard test plant for recovery of the virus from infected plants and for property studies.

All inoculations were made in a greenhouse where temperatures ranged from 13° to 19° C. The methods followed were essentially those described in recent papers (*16*, *19*). Mechanical inoculations were made by dusting the leaves with 600-mesh, powdered carborundum (*10*) and lightly rubbing with absorbent cotton dipped in juice from a diseased plant.

The cabbage black ring virus is transmissible to healthy cabbage seedlings by mechanical inoculation only when carborundum (*10*) is used. As a result of numerous tests, the incubation period was determined as ranging from 9 to 21 days.

Since Essig (*5*) found that the cabbage aphid (*Brevicoryne brassicae* (L.)) and the green peach aphid (*Myzus persicae* (Sulzer)) breed naturally on cultivated and wild crucifers in California, it seemed desirable to test these two aphid species under greenhouse conditions.

Following the procedure outlined in a recent paper (*19*), noninfective

cabbage and green peach aphids [6] were fed on recently infected cabbage plants for 24 to 48 hours, after which they were transferred in lots of approximately 20 aphids each to healthy cabbage seedlings. After 24 hours, all plants were sprayed with nicotine sulphate solution. Healthy cabbage seedlings infested with noninfective aphids and noninoculated plants free from aphids served as controls.

Of 20 cabbage plants infested with viruliferous cabbage aphids, 15 showed typical symptoms of the disease in 9 to 16 days. When infective green peach aphids were placed on 20 cabbage plants, 17 plants were infected, the incubation period ranging from 10 to 14 days. The virus was recovered from all infected plants by mechanical inoculation. None of the controls became diseased.

HOST RANGE

Other crucifers in addition to cabbage which are subject to natural infection by the cabbage black ring virus, as proved by mechanical inoculation in the greenhouse with expressed juice from representative field samples, include brussels sprouts, sprouting broccoli, and cauliflower. The chlorotic and necrotic ring symptoms characteristic of infection on cabbage were also observed on all of these hosts.

Numerous commercial varieties of white and red cabbage were tested in the greenhouse by mechanical inoculation to determine their degree of susceptibility. In addition to the variety Winter Colma, previously referred to as the standard test plant, all varieties proved to be highly susceptible. The number of plants of any particular variety to be tested varied from 20 to 200. Symptoms shown by infected plants and the length of the incubation period, irrespective of variety, were in close agreement with those of the variety Winter Colma.

The following varieties of cabbage were tested: White—Allhead Early, All Seasons, Charleston or Large Wakefield, Copenhagen Market (two selections), Danish Ballhead (two selections), Early Drumhead Savoy, Early Dwarf Flat Dutch, Early Jersey Wakefield, Early Winningstadt, Extra Early Express Round, Glory of Enkhuizen, Golden Acre (two selections), Hollander, Improved Globe, Large American Drumhead, Marion Market, Midseason Market, Penn State Ballhead, Perfection Drumhead Savoy, Premium Late Flat Dutch, San Francisco Market, Stone Mason, Succession, Surehead, Volga, Wisconsin All Seasons, and Wisconsin No. 8; red—Mammoth Rock Red (two selections), and Round Red Dutch.

As compared with certain other crucifer viruses which occur in California (*15*, *16*, *19*), the cabbage black ring virus has a more cosmopolitan host range, as shown in table 1. In the family Cruciferae, nine species of Brassica, two species of Matthiola, and seven other genera showed systemic infection. The symptoms obtained on certain crucifers are shown in figures 4 and 5.

Infection was also obtained on plants representing 16 genera and 19 species in 11 additional families, as shown in table 1. Symptoms produced on certain of these hosts are shown in figures 4 and 6.

The virus was recovered from all infected plants with the exception of rhubarb, lambsquarters, sowbane, and spinach. No valid reason

[6] Aphids identified by E. O. Essig, as noninfective cabbage and green peach aphids, were kindly supplied by Dr. H. H. P. Severin and Dr. J. H. Freitag, Division of Entomology and Parasitology, University of California.

can be assigned for failure to recover the virus from these plants on either cabbage or Turkish tobacco.

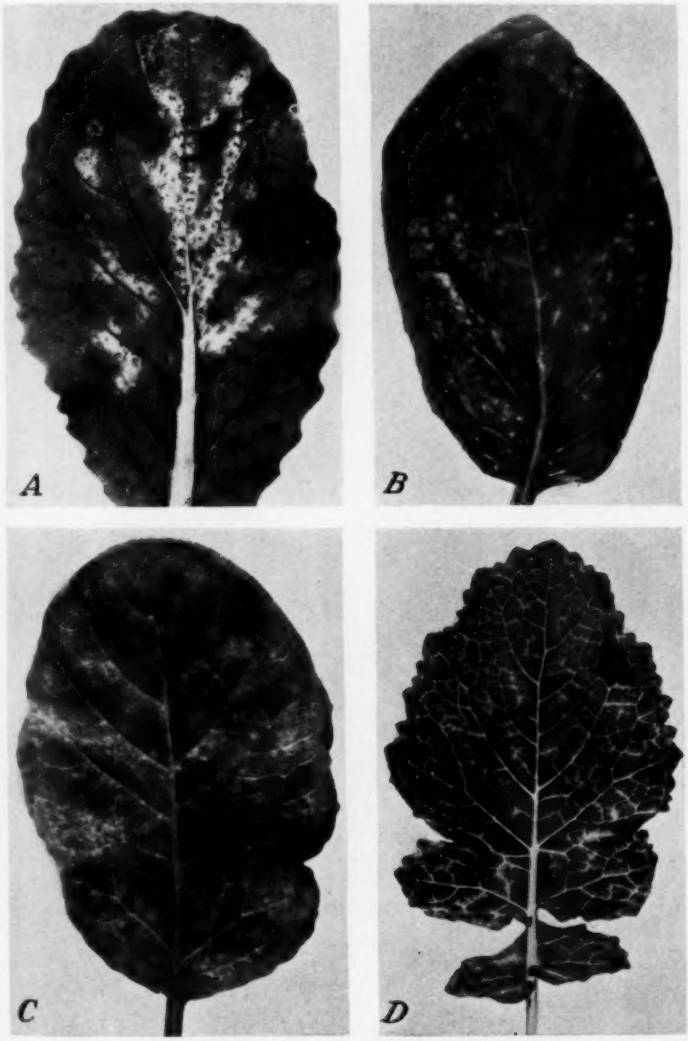


FIGURE 4.—Symptoms produced by the black ring virus on certain host plants after mechanical inoculation in the greenhouse at 13° to 19° C. on: *A*, Chinese cabbage or pe-tsai; *B*, Bloomsdale spinach; *C*, Chinese radish; *D*, Purple Top White Globe turnip.

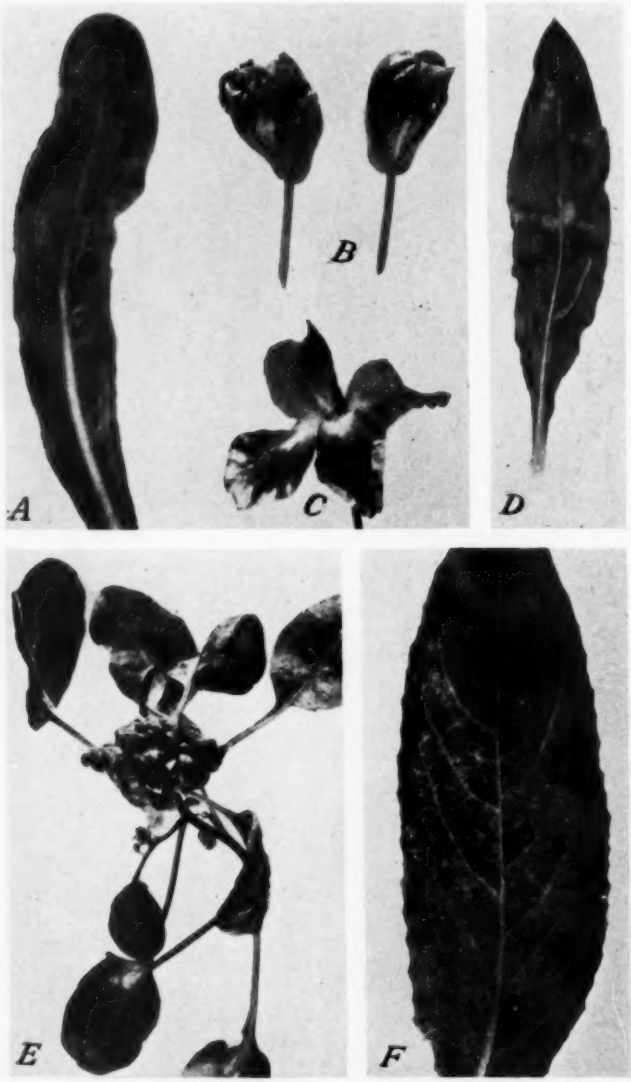


FIGURE 5.—Symptoms produced by the black ring virus on certain ornamental crucifers after mechanical inoculation in the greenhouse at 13° to 19° C.: *A*, *B*, *C*, Leaf, buds, and flower of Fiery Blood Red annual stock; *D*, wallflower; *E*, Virginian stock; *F*, dames violet.

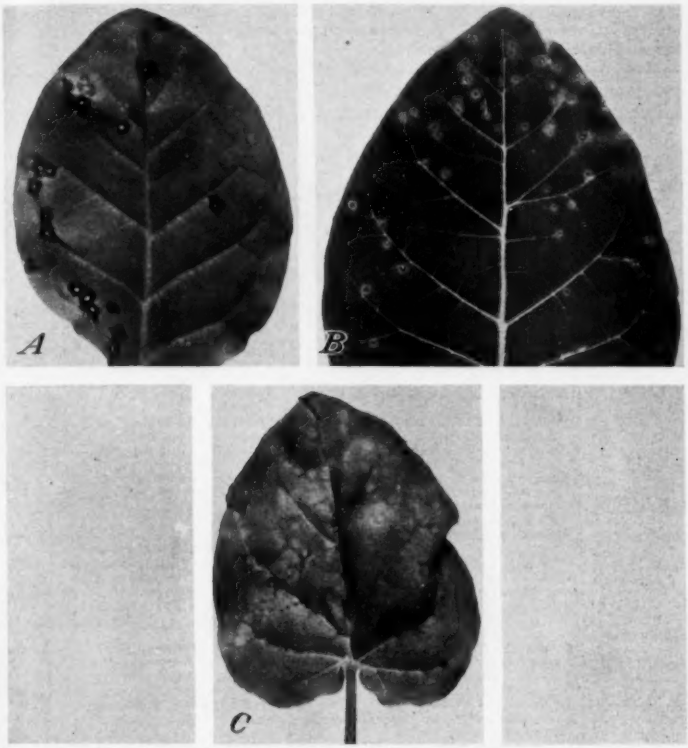


FIGURE 6.—Symptoms produced by the black ring virus on species of *Nicotiana* after mechanical inoculation in the greenhouse at 13° to 19° C.: *A*, Local lesions on *N. tabacum* var. Turkish; *B*, var. White Burley; *C*, *N. glutinosa*.

TABLE 1.—*Plants susceptible to the cabbage black ring virus, as indicated by mechanical inoculation of greenhouse-grown seedlings, and symptoms characteristic of infection*

Family	Species and common name	Symptoms produced
Cruciferae	*Brassica oleracea* L. var. *acephala* DC. (kale).	Systemic. Chlorotic rings. Later, tan, necrotic, irregular-shaped lesions.
	B. oleracea L. var. *gemmifera* DC. (Brussels sprouts).	Systemic. Chlorotic rings which later become necrotic.
	B. oleracea L. var. *botrytis* L. (cauliflower—var. February).	Systemic. Chlorotic rings with marked leaf chlorosis in later stages of infection.
	B. oleracea L. var. *botrytis* L. (asparagus or sprouting broccoli—var. Italian Green Sprouting).	Systemic. Chlorotic rings which later become necrotic.
	B. oleracea L. var. *caulorapa* DC. (kohlrabi—var. Early White Vienna).	Do.
	B. pe-tsai Bailey (pe-tsai)	Do.
	B. napus L. (rape)	Systemic. Chlorotic rings.
	B. campestris L. var. *napobrassica* DC. (rutabaga).	Do.
	B. rapa L. (turnip—var. Purple Top White Globe).	Systemic. Chlorotic rings which later become necrotic. The necrotic lesions are largely confined to the older leaves.
	B. juncea Coss. (leaf mustard)	Systemic. Chlorotic rings.
	B. alba (L.) Boiss. (white mustard)	Systemic. Chlorotic rings confined to inoculated leaves, with vein clearing on new, inner leaves.

TABLE 1.—*Plants susceptible to the cabbage black ring virus, as indicated by mechanical inoculation of greenhouse-grown seedlings, and symptoms characteristic of infection*—Continued

Family	Species and common name	Symptoms produced
Cruciferae	*B. adpressa* Boiss	Systemic. Chlorotic rings.
	B. arvensis (L.) Ktze. (charlock)	Systemic. Chlorotic rings which later become necrotic. Infection caused early death of plants.
	Capsella bursa-pastoris (L.) Medic. (Shepherdspurse).	Systemic. Chlorotic rings and downward curling of leaf.
	Cheiranthus cheiri L. (wallflower)	Systemic. Chlorotic rings, with some fusion.
	Matthiola bicornis DC. (evening scented stock).	Systemic. A bleaching type of mottling.
	M. incana R. Br. (Brompton stock)	Systemic. Chlorotic rings, coarse vein clearing, and some leaf distortion.
	M. incana R. Br. var. *annua* Voss (annual stock).	Systemic mottling causing rapid bleaching of leaf and leaving small, dark-green islands. Breaking of flowers occurred.
	Hesperis matronalis L. (dames violet)	Systemic. Vein clearing of young, inner leaves followed by development of chlorotic and necrotic lesions.
	Malcomia maritima R. Br. (Virginian stock).	Systemic. Mottling and rosetting of new growth.
	Radicula nasturtium-aquaticum Brit. & Rend. (water cress).	Systemic. Chlorotic rings.
	Lunaria annua L. (honesty)	Systemic. Mottling and bronzing of leaf, with vein necrosis.
	Raphanus sativus L. var. *longipinnatus* Bailey (Chinese radish).	Systemic. Chlorotic rings.
Begoniaceae	*Begonia semperflorens* Link and Otto (fibrous-rooted begonia).	Do.
Boraginaceae	*Myosotis alpestris* Schmidt (forget-me-not).	Systemic. Chlorotic and necrotic lesions on leaves and petioles.
Caryophyllaceae	*Stellaria media* (L.) Cyrill (chickweed)	Systemic. Chlorotic rings and mottling.
Chenopodiaceae	*Chenopodium album* L. (lambsquarters or white pigweed).	Systemic. Chlorotic to yellow rings 1/16 inch in diameter which later enlarge to 3/16 inch. The pale to yellow centers surrounded by narrow, chlorotic band or halo.
	C. murale L. (sowbane or nettle-leaf goosefoot).	Systemic. Chlorotic rings which later turn yellow with a green edge.
	Spinacia oleracea L. (spinach—var. Bloomsdale)	Systemic. Chlorotic rings which later turn a bright yellow and fuse, giving the leaf a yellowed appearance.
Compositae	*Chrysanthemum coronarium* L. (annual marguerite).	Systemic. Vein clearing.
	Dimorphotheca aurantiaca DC. (winter Cape-marigold).	Systemic. Chlorotic rings become necrotic around the edges.
	Senecio cruentus DC. (hybrid cineraria)	Systemic. Vein necrosis and chlorosis of young, inner leaves.
	Zinnia elegan Jacq. (zinnia or youth-and-old-age).	Systemic. Vein clearing.
Dipsacaceae	*Scabiosa atropurpurea* L. (mourning bride or pincushion flower).	Systemic. Vein clearing.
Papaveraceae	*Papaver nudicaule* L. (Iceland poppy)	Systemic. Leaves mottled and containing necrotic lesions surrounded by chlorotic halos. Necrotic streaks on petioles.
Polygonaceae	*Rheum rhaponticum* L. (rhubarb)	Systemic. Chlorotic rings.
Resedaceae	*Reseda odorata* L. (mignonette)	Do.
Solanaceae	*Nicotiana glutinosa* L	Systemic. Chlorotic rings at first, followed by necrotic lesions which appeared on older, outer leaves only.
	N. langsdorffii Weinm	Systemic. Mottling and tan, necrotic lesions.
	N. tabacum L. (tobacco—var. Turkish)	Local, tan necrotic lesions on inoculated leaves only. Not systemic.
	——var. White Burley	Chlorotic rings with necrotic centers on inoculated leaves only. Not systemic.
	Petunia hybrida Hort. (petunia)	Systemic. Chlorotic rings. Curling and puckering of leaves and stunting of plant.
Verbenaceae	*Verbena hybrida* Voss (garden verbena)	Systemic. Chlorotic rings with necrosis of veins and midrib.

No infection was obtained on other plants representing 26 families, 49 genera, and 53 species, as follows:

Boraginaceae:
Alkanet (*Anchusa officinalis* L.)
Common heliotrope (*Heliotropium peruvianum* L.)

Campanulaceae: Canterbury-bells (*Campanula medium* L.)
Caryophyllaceae:
Sweet-william (*Dianthus barbatus* L.)
Babysbreath (*Gypsophila paniculata* L.)
Chenopodiaceae:
Sugar beet (*Beta vulgaris* L.)
Swiss chard (*B. vulgaris* var. *cicla* Moq.)
Compositae:
Head lettuce (*Lactuca sativa* L. var. *capitata* Hort.) var. New York and Tom Thumb
Dandelion (*Taraxacum officinale* Weber)
Transvaal daisy (*Gerbera jamesonii* Hook. var. *transvaalensis* Hort.)
Shasta daisy (*Chrysanthemum maximum* Ram.)
Dahlia sp.
China-aster (*Callistephus chinensis* Nees) var. Giant Branching White, wilt resistant
English daisy (*Bellis perennis* L.)
French marigold (*Tagetes patula* L.)
Gaillardia pulchella Foug. var. *picta* Gray
Convolvulaceae: Morning-glory (*Ipomoea purpurea* Roth)
Cruciferae:
Brassica integrifolia O. E. Schulz var. *chevalieri* R. Porteres
Rockcress (*Arabis albida* Stev.)
Goldentuft (*Alyssum saxatile* L.)
Radish (*Raphanus sativus* L.) var. White Icicle
Cucurbitaceae: Cucumber (*Cucumis sativus* L.)
Euphorbiaceae: Castor-bean (*Ricinus communis* L.)
Geraniaceae: Storksbill (*Pelargonium zonale* Willd.)
Gramineae:
Corn (*Zea mays* L.) var. Golden Bantam
Oat (*Avena sativa* L.)
Labiatae:
Flowering sage (*Salvia farinacea* Benth.)
Bugleweed (*Ajuga reptans* L. var. *rubra* Hort.)
Leguminosae:
Garden pea (*Pisum sativum* L.) var. Alderman
Broadbean (*Vicia faba* L.)
Alfalfa (*Medicago sativa* L.) var. Arabian, Baltic, California Common, Cossack, Grimm, Hardistan, Hemet, and Modoc
Lobeliaceae: Lobelia (*Lobelia hybrida* Hort.)
Malvaceae:
Cheeseweed (*Malva parviflora* L.)
Hollyhock (*Althaea rosea* Cav.)
Onagraceae:
Clarkia elegans Dougl.
Godetia grandiflora Lindl.
Papaveraceae: Oriental poppy (*Papaver orientale* L.)
Plumbaginaceae: Everlasting or sea-lavender (*Statice latifolium* Kuntze)
Ranunculaceae:
Rocket larkspur (*Delphinium ajacis* L.)
Hybrid delphinium (*D. cultorum* Voss)
Rosacea: *Geum chiloense* Balb.
Scrophulariaceae:
Snapdragon (*Antirrhinum majus* L.)
Pentstemon or beardtongue (*Pentstemon barbatus* Nutt.)
Solanaceae:
Solanum aviculare Forst.
Potato (*S. tuberosum* L.)
Eggplant (*S. melongena* L. var. *esculentum* Nees)
Tomato (*Lycopersicum esculentum* Mill. var. *vulgare* Bailey) var. Early Santa Clara Canner
Currant tomato (*L. pimpinellifolium* Dunal)
Nicotiana rustica L. var. *humulis* Schrank
Jimsonweed (*Datura stramonium* L.)
Tropaeolaceae: Garden nasturtium (*Tropaeolum majus* L.)
Umbelliferae: Celery (*Apium graveolens* L.) var. Golden Self Blanching
Valerianaceae: Garden heliotrope (*Valeriana officinalis* L.)
Violaceae: Pansy (*Viola tricolor* L.)

PROPERTIES OF THE VIRUS

Three properties of the black ring virus of cabbage were studied, as shown in table 2. The virus was infectious after being stored for 48 hours at 22° C. but was inactivated after 72 hours. Of 25 cabbage plants inoculated with virus extract heated for 10 minutes at 57°, only 1 plant became infected. No infection resulted from inoculations with virus extract heated at 59°. It is concluded, therefore, that the inactivation temperature lies between 57° and 59°. The virus has a dilution tolerance of approximately 1 to 700, beyond which infection did not occur.

In their studies of the properties of a cabbage mosaic virus in Wisconsin, Larson and Walker (7) reported inactivation at 55° C. for a 10-minute exposure. A dilution of 1 to 1,000 and storage for 2 days at 20° to 22° did not inactivate the virus.

TABLE 2.—*Longevity in vitro, inactivation temperature, and tolerance to dilution of the cabbage black ring virus*

[Five trials made with 25 plants each in all instances]

LONGEVITY IN VITRO, 22° C.

Aged (hours)	Plants infected	Aged (hours)	Plants infected	Aged (hours)	Plants infected	Aged (hours)	Plants infected
	Number		*Number*		*Number*		*Number*
0	25	8	13	48	4	96	0
4	20	24	19	72	0	120	0

INACTIVATION TEMPERATURE (10 MINUTES)

Temperature (°C.)	Plants infected	Temperature (°C.)	Plants infected	Temperature (°C.)	Plants infected	Temperature (°C.)	Plants infected
	Number		*Number*		*Number*		*Number*
Not heated	25	55	15	59	0	65	0
50	23	57	1	60	0	70	0

TOLERANCE TO DILUTION

Dilution	Plants infected	Dilution	Plants infected	Dilution	Plants infected	Dilution	Plants infected
	Number		*Number*		*Number*		*Number*
0	20	1:50	4	1:500	1	1:2,000	0
1:10	14	1:100	5	1:700	3		
1:25	8	1:200	4	1:1,000	0		

DESCRIPTION OF THE BLACK RING VIRUS OF CABBAGE

Transmissible by mechanical inoculation with expressed juice and powdered carborundum at temperatures of 13° to 19° C. Transmitted in greenhouse tests by means of cabbage aphid (*Brevicoryne brassicae* (L.)) and green peach aphid (*Myzus persicae* (Sulzer)). Incubation period 9 to 21 days. Resistance to aging in vitro between 48 and 72 hours. Inactivation temperature at or near 59° C. for 10-minute exposure. Tolerance to dilution approximately 1 to 700. Cabbage (*Brassica oleracea* L. var. *capitata* L.) and numerous other vegetable and ornamental crucifers susceptible. On cabbage, symptoms consist of numerous chlorotic rings, some becoming necrotic with age. Occasionally necrotic streaking of veins. In the field, symptoms are conspicuous only on older leaves.

COMPARISON OF THE BLACK RING VIRUS WITH CERTAIN OTHER CRUCIFER VIRUSES

The viruses of cauliflower mosaic (*16*), Chinese cabbage mosaic (*19*) turnip mosaic (*17*), mild and severe annual stock mosaic (*15*)[7], and radish mosaic [7] were tested simultaneously with the black ring virus in the greenhouse in parallel series of inoculations to various plants. For comparative purposes, the more important symptom differences on certain differential hosts are presented.

On Winter Colma cabbage, the cauliflower mosaic virus (*16, fig. 3, C*) causes vein clearing; the Chinese cabbage mosaic virus (*19, fig. 2, C*), coarse, yellowish, vein banding; the turnip mosaic virus (*16, fig. 5, A; 17, fig. 2*), chlorotic rings with necrotic edges; and the radish mosaic virus, large chlorotic rings, some of which later become necrotic, but differ in color, size, and shape from those produced by the black ring virus. No infection of cabbage was obtained with either the mild or the severe mosaic virus of annual stock.

On February cauliflower, the cauliflower mosaic virus caused vein clearing, followed by vein banding and necrotic spotting (*16, fig. 1, A, B, C*); the Chinese cabbage mosaic virus, scattered chlorotic rings with dark-green centers (*19, fig. 2, B*); the turnip mosaic virus, pale-green to yellow lesions (*17, fig. 5, B*); and the radish mosaic virus, diffuse chlorotic lesions. No infection of cauliflower was obtained with either the mild or the severe mosaic virus of annual stock.

On Chinese cabbage, or pe-tsai, the black ring virus, as already shown, caused chlorotic rings, some of which later became necrotic. The cauliflower mosaic virus (*16*) caused vein clearing; the Chinese cabbage mosaic virus (*19, fig. 1, A, B, C*), vein clearing and mottling; the turnip mosaic virus (*17*), coarse yellow vein banding and leaf distortion; the mild mosaic virus of annual stock, a fine to medium type of mottling; and the radish mosaic virus, chlorotic and necrotic lesions and vein necrosis on inoculated leaves only. The severe mosaic virus of annual stock did not cause infection.

On Purple Top White Globe turnip, the black ring virus induced chlorotic rings, some of which were necrotic. The cauliflower mosaic virus (*16*) caused vein clearing; the Chinese cabbage mosaic virus (*19*), a fine type of vein clearing and mottling; the turnip mosaic virus, vein clearing followed by mottling with raised islands, crinkling, and stunting of plants; the mild and severe mosaic viruses of annual stock, a fine to medium type of mottling; and the radish mosaic virus, mottling with severe distortion of leaves.

Numerous unsuccessful attempts were made to infect White Icicle radish with the black ring virus. Paralleling this is the fact that the turnip (*17*) and severe annual stock mosaic viruses do not infect radish. On the other hand, the cauliflower mosaic virus (*16*) caused vein clearing; the Chinese cabbage mosaic virus (*19, fig. 2, C*), vein clearing and mottling; the mild mosaic virus of annual stock, a fine to medium type of mottling; and the radish mosaic virus, coarse mottling and raised dark-green islands on a chlorotic to yellow background.

The black ring virus caused mottling of the leaves and flower breaking on Fiery Blood Red annual stock; the cauliflower mosaic virus (*16, fig. 4, A, B, C*), coarse vein clearing but no flower breaking; the Chinese cabbage mosaic virus (*19*), mottling of leaves and flower

[7] The mild and severe mosaic diseases of annual stock and a mosaic disease of radish are fully described in papers now in press.

breaking; the turnip mosaic virus (*17*), coarse vein clearing, diffuse mottling, and flower breaking; the mild annual stock mosaic virus, a fine type of mottling and flower breaking; and the severe mosaic virus of annual stock, very coarse, conspicuous leaf mottling and flower breaking. No infection was obtained with the radish mosaic virus.

SUMMARY

A virus disease of cabbage, cauliflower, and other cultivated crucifers, designated as black ring, is described in this paper.

The disease occurs chiefly in the cool, coastal valleys of California during the winter months and is uncommon in the summer.

The disease causes marked damage to the older, outer leaves. It is characterized by chlorotic lesions, many of which become partially or entirely necrotic with age. The symptoms are most conspicuous on the under surface of the leaf.

Under greenhouse conditions, the black ring virus has been transmitted to healthy cabbage plants by means of the cabbage and green peach aphids. It is also readily transmissible by juice inoculations with powdered carborundum.

The inactivation temperature of the virus lies between 57° and 59° C. The virus withstands aging in vitro at 22° C. for 48 hours, and has a tolerance to dilution of 1 to 700.

All tested varieties of cabbage proved susceptible.

In the family Cruciferae, systemic infection was obtained on kale, Brussels sprouts, sprouting broccoli, kohlrabi, turnip, pe-tsai, charlock, dames violet, cauliflower, rape, rutabaga, leaf or Chinese mustard, white mustard, *Brassica adpressa*, shepherdspurse, wallflower, Brompton stock, water cress, Chinese radish, evening scented stock, annual stock, and honesty.

Infection was also obtained on plants representing 11 additional families, as follows: Rhubarb, lambsquarters, sowbane, spinach, chickweed, mignonette, fibrous-rooted begonia, garden verbena, petunia, Iceland poppy, *Nicotiana glutinosa*, *N. langsdorffii*, *N. tabacum*, mourning bride, annual marguerite, zinnia, winter Cape-marigold, cineraria, and forget-me-not.

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SOIL-FERTILITY STANDARDS FOR GROWING NORTHERN CONIFERS IN FOREST NURSERIES[1]

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INTRODUCTION

In nursery management, the state of soil fertility is one of the most important factors that affect the development of seedlings. A deficiency of any of the essential nutrients or an unbalanced ratio has a far-reaching influence on the entire process of metabolism. The ill effects of malnutrition are reflected in abnormal size and color of leaves, inadequate root systems, unbalanced top-root ratio, thin cell walls and porous structure of wood tissue, underdeveloped nuclei in the parenchymatic cells, and other anatomical and physiological abnormalities (*4*, *5*, *8*, *9*).[3] Ultimately, all of these ailments result in a decreased resistance of seedlings to drought, frost, and infectious diseases.

CONTENT OF NUTRIENTS AND ASSOCIATED CONDITIONS IN VIRGIN FOREST SOILS

Recently cleared but not burned-over forest land, which previously supported a mature or nearly mature stand of satisfactory growth, when used as a nursery site, always produces at least one crop of vigorous seedlings of the same species that originally grew on the area. This is common knowledge acquired from many years of experience, particularly with temporary nurseries in Europe. Consequently, the nutrient content of productive forest soils is a satisfactory criterion for establishing soil-fertility standards in forest nurseries. Based on this fact, a detailed study of the chemical properties of soils supporting normally developed stands of jack pine (*Pinus banksiana* Lamb.), red pine (*P. resinosa* Ait.), northern white pine (*P. strobus* L.), white spruce (*Picea glauca* (Moench) Voss), and balsam fir (*Abies balsamea* (L.) Mill.), and mixed stands of northern hardwoods (*Acer saccharum* Marsh., *Tilia americana* L., *Betula lutea* Michx. f., etc.,) has been carried out. The minimum age of the jack pine stands was 25 years and that of the other species 40 years.

About 500 soil samples were collected from the following areas: Chippewa and Superior National Forests and Cloquet Experimental Forest in Minnesota; Chequamegon and Nicolet National Forests, the Wisconsin River Valley from Wisconsin Dells to Stevens Point, and Brule, Northern, and American Legion State Forests in Wisconsin; Ottawa, Hiawatha, and Huron National Forests, Manistee pur-

[1] Received for publication May 31, 1938. Issued February 1939. Contribution from the Soils Department, Wisconsin Agricultural Experiment Station, in cooperation with the Wisconsin Department of Conservation. The work was supported by Works Progress Administration funds.

[2] Credit is due S. F. Buran, senior chemist, who was engaged in laboratory analyses connected with this study. H. M. Galloway and J. G. Cady assisted in field work, statistical treatment of the results, and preparation of the manuscript.

[3] Italic numbers in parentheses refer to Literature Cited, p. 951

chase unit, and the Higgings Lake region in Michigan; Hoosier purchase unit in Indiana; Gardner purchase unit in Missouri; and the Wayne purchase unit in Ohio.

The results of analyses for pH value (*10*), silt and clay fractions (*2*), base exchange capacity (*3*), total nitrogen (*1*), nitrates (*6*), ammonia (*7*), available phosphorus (*11*), available potassium (*12*), replaceable calcium, and replaceable magnesium (*3*) are given in tables 1, 2, and 3.

TABLE 1.—*Silt, clay, and organic-matter content and base-exchange properties of soils of various forest sites*

Site	Silt, 0.05–0.005 mm	Clay, less than 0.005 mm	Total fine separates	Organic matter [1]	Base-exchange capacity per 100 g	Total replaceable bases per 100 g	Base saturation
	Percent	*Percent*	*Percent*	*Percent*	*Milli-equivalents*	*Milli-equivalents*	*Percent*
Barrens	3.8±0.6	1.1±0.1	4.9	1.93	2.18±0.2	1.5	68.8
Jack pine	7.4± .4	2.0± .2	9.4	3.76	4.30± .2	2.6	60.5
Red pine	10.6±1.3	3.4± .9	14.0	4.68	8.26± .4	4.3	52.0
White pine	12.0± .9	6.4± .8	18.4	5.25	9.60± .5	6.7	69.8
White spruce	25.3±2.4	28.6±1.5	53.9	7.19	15.10± .9	8.5	56.3
Hardwoods	28.8±2.1	24.4±1.8	53.2	6.85	16.30± .5	12.9	79.1

[1] Approximate figures for "loss on ignition," arrived at largely on the basis of total nitrogen determinations.

TABLE 2.—*Content of nitrate and ammonia nitrogen and its relation to the content of total nitrogen in soils of different forest sites*

Site	Medians for—		Arithmetic means of—			Total N	Relation of available N to total N	Approximate level of available N per acre
	Nitrate N	Ammonia N	Nitrate N	Ammonia N	Total available N			
	P. p. m.	*P. p. m.*	*P. p. m.*	*P. p. m.*	*P. p. m.*	*P. p. m.*	*Percent*	*Pounds*
Barrens	Tr.	Tr.	0.8	2.2	3.0	430	0.69	9
Jack pine	Tr.	5	2.3	6.0	8.3	1,040	.79	20
Red pine	Tr.	11	1.6	9.9	11.5	1,190	.96	29
White pine	Tr.	10	2.1	11.8	13.9	1,420	.98	35
White spruce	2	24	2.9	19.0	21.9	2,570	.85	44
Hardwoods	10	Tr.	11.5	5.7	17.2	2,210	.78	35

TABLE 3.—*Reaction and content of nutrients in soils of different forest sites*

Site	Reaction	Total N	Available P_2O_5 per acre	Available K_2O per acre	Replaceable Ca per 100 g	Replaceable Mg per 100 g
	pH	*Percent*	*Pounds*		*Milli-equivalents*	*Milli-equivalents*
Barrens	6.30±0.12	0.043±0.005	25.8± 2.5	66.9± 9.5	1.1±0.2	0.3±0.1
Jack pine	5.60± .07	.104± .003	37.5± 6.3	92.0± 7.24	1.9± .1	.5± .1
Red pine	5.33± .11	.119± .004	43.9± 2.7	152.8±10.2	3.2± .2	.9± .1
White pine	5.40± .10	.142± .005	82.8± 9.6	192.0±11.0	5.0± .4	1.4± .2
White spruce	5.19± .09	.257± .010	98.3± 9.9	287.4±13.3	6.1± .7	2.1± .2
Hardwoods	6.07± .07	.221± .015	175.8±13.1	301.7±20.6	10.3± .8	2.4± .1

The mean values presented in these tables were derived from 30 to 40 single figures expressing the fertility of the upper 8-inch layer of the

soil profile. These figures for individual soil profiles were obtained by the analysis of soil samples collected from the separate soil horizons, such as litter (A_{00}), duff (A_0), humic layer (A_1), and leached podsolic layer (A_2). The average thickness of each horizon, its volume weight, and its content of nutrients were considered in computing the total fertility of each 8-inch layer. An example will illustrate the method employed. A layer of litter and duff 2 inches in thickness contains 300 p. p. m. of available phosphorus pentoxide, whereas the 6-inch layer of mineral soil underlying the duff contains 40 p. p. m. of phosphorus pentoxide. The weight per acre of 2-inch duff and 6-inch loam layers is 240,000 and 2,000,000 pounds, respectively. Thus, the content of phosphorus pentoxide in the duff layer is $240{,}000 \times 0.0003$, or 72 pounds, whereas the content of the same constituent in mineral soil is $2{,}000{,}000 \times 0.00004$, or 80 pounds. Hence, the total amount of available P_2O_5 in a soil layer 8 inches deep is $72+80$ or 152 pounds per acre, or per 2,240,000 pounds of soil. This corresponds to $152 \times 1{,}000{,}000 \div 2{,}240{,}000$, or 67 p. p. m. This is the content of phosphorus pentoxide which will be found in the top 8 inches of nursery soil after the analyzed layers have been mixed by plowing and disking or by rototilling.

The analysis of soil was not extended beyond a depth of 8 inches since the soil at greater depths does not affect the nutrition of young seedlings in the nursery. The standard deviations for establishing standard errors were calculated by dividing the sum of squared deviations by $n-1$. The data for available nitrogen given in table 2 cannot be regarded as having more than a relative value, since the amounts of ammonia and nitrates vary greatly with the season and other conditions. Because of this variability, medians, as well as arithmetic means, are reported for these two factors.

Table 4 presents in round numbers the approximate desirable levels or ranges of different soil factors, which, with the qualifications discussed below, may serve as standards in the maintenance of nursery soil fertility. The use of these standards has been tested with satisfactory results in about 30 Federal, State, and private forest nurseries located in Wisconsin and several neighboring States.

The barren soils were analyzed simply in order to determine the lowest level of soil fertility. The study of hardwood soils was not confined to any particular species, and requires additional analyses. Consequently, no data for these two sites are given in table 4.

TABLE 4.—*Standards of fertility for nursery blocks raising different species of trees*

Species	Reaction	Base-exchange capacity per 100 g	Total N	Approximate level of available N per acre	Available P_2O_5 per acre	Available K_2O per acre	Replaceable Ca per acre	Replaceable Mg per acre
	pH	*Milliequivalents*	*Percent*	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*
Jack pine	5.6	5.0	0.10	20	40	100	1,000	150
Red pine	5.4	8.0	.12	30	50	150	1,500	300
White pine	5.4	10.0	.14	35	80	200	2,500	450
White spruce	5.2	15.0	.25	45	100	275	3,000	600

The typological investigations in Europe, as well as greenhouse studies at this Station, have indicated that the standards established for red pine and white spruce may be safely applied to Scotch pine (*Pinus sylvestris* L.) and Norway spruce (*Picea excelsa* Link).

PRINCIPLES OF NURSERY SOIL IMPROVEMENT

Ordinarily, soil improvement involves a period of several years and follows a definitely outlined program. Only a few nurseries may be brought to a satisfactory level of fertility by means of a single application of fertilizer and absorbing materials. The time element is particularly important when a nursery soil has a low content of exchange material and total nitrogen, or considerable deviation of pH value from the desired state. In many instances even the correction of potassium and phosphorus deficiency may require a period of several years.

In outlining a soil-improvement program it is important to keep two conditions in mind. The first is the undesirability of introducing large quantities of raw organic remains and mineral fertilizers, for such introduction may bring about a deficiency of available nitrogen and a toxicity of soil solution due to a high concentration of salts. On the other hand, undue delay in attaining full productive capacity of the nursery soil may result in financial losses caused by waste of unabsorbed fertilizers and may lead to production of underdeveloped stock. Therefore, expert judgment is necessary in determining the time needed to complete the soil-building program.

It is important to realize that the nutrient requirements of seedlings cannot be satisfied by a purely arithmetic balance between the mineral constituents of fertilizers and their consumption by stock, but only through complex physiochemical and biological processes. The growth of seedlings in a soil containing unabsorbed fertilizer salts is different from the growth in medium in which the salts have become incorporated into organic matter by means of chemical and biological reactions. In the first case, the nutrient medium is characterized by equal amounts of cations and anions, great periodic changes in concentration of soil solution due to fluctuations of soil moisture, and a number of other conditions which are not favorable to the normal nutrition of forest trees. In the second case, most of the nutrients are present as complex organic compounds, and the absorbed cations predominate in the soil since the anions are largely eliminated by leaching. It is evident, therefore, that the soil-building process is not completed merely by the addition of a certain amount of peat and mineral fertilizers, but only when these materials have undergone a certain period of "composting" and have become incorporated with the soil through microbiological activity and exchange reactions.

GENERAL DIRECTIONS FOR ADJUSTMENT OF SOIL CONDITIONS

The adjustment of pH value of soil has recently received considerable attention in both agricultural and phytopathological literature. The correction of colloid content, base exchange capacity, and total nitrogen has been previously outlined[4] (*13*, *16*).

[4] WILDE, S. A. ADJUSTMENT OF SOIL FERTILITY IN CONIFEROUS NURSERIES. Wis. State Conserv. Dept. in Coop. with Wis. Agr. Col. Tech. Notes 19, 9 pp. 1937. [Mimeographed.]

Under favorable conditions of nursery soil, the activity of microorganisms provides a sufficient amount of available nitrogen by converting protein compounds into ammonia and nitrates. The usual content of nitrate and ammonia nitrogen together in a nursery soil amounts to about 1 percent of the total nitrogen [4] (*9*), i. e., about 40 pounds per acre, if nursery soil contains 0.2 percent of total nitrogen. Under such well-balanced conditions there is no need to apply available nitrogen in the form of commercial fertilizers. In many instances, however, the amount of available nitrogen released by soil organisms may not be sufficient for seedling growth. This may be due to crowded seedbeds, the presence of raw organic matter high in carbon, or soil unfavorable for microbiological activity. The last-named condition may be expected particularly in nursery soils which have been disinfected with toxic substances. In such cases, the content of available nitrogen should be increased by the application of ammonia or nitrate fertilizers.

There is at hand no simple procedure for the determination of the exact amount of mineral nitrogen fertilizer to apply, since this depends upon a great variety of factors. Fortunately, deficiency of nitrogen is readily manifested by discoloration of foliage, and consequently the application of nitrogen fertilizers is usually dictated by the appearance of the stock. A good rule to follow in nitrogen fertilization is to apply too little rather than too much of the salt. A deficiency may be corrected by a second application. An excess results in planting stock of reduced vigor.

Table 5 gives the approximate range of annual applications of nitrogen salts in the form of ammonium sulphate which is likely to be required by different tree species.

TABLE 5.—*Approximate range (pounds per acre) of annual applications of elemental nitrogen or of nitrogen salt in the form of ammonium sulphate likely to be required by different tree species*

Forms of nitrogen	Jack pine	Norway pine	White pine	Spruce
	Pounds per acre	*Pounds per acre*	*Pounds per acre*	*Pounds per acre*
Elemental	10–20	20–40	30–50	40–60
20 percent ammonium sulphate	50–100	100–200	150–200	200–300

The analyses of the productive forest soils show that the nitrogen-phosphoric acid-potash ratio under natural conditions is about 1-2-5 for all four coniferous species studied. Experience in the greenhouse, as well as in the nursery, indicated that the nitrogen content should not greatly exceed the ratio of 2-2-5, if vigorous, well-balanced planting stock is to be produced. This means that in a soil with a phosphoric acid content of 40 pounds and a potash content of 100 pounds per acre, the allowable maximum of available nitrogen is 40 pounds per acre. Further studies are under way to confirm the extent of applicability of this ratio under a wider range of conditions.

The deficiency of phosphate, potash, and replaceable bases is corrected either through the application of organic remains or mineral

[4] WILDE, S. A. ADJUSTMENT OF SOIL FERTILITY IN CONIFEROUS NURSERIES. Wis. State Conserv. Dept. in Coop. with Wis. Agr. Col. Tech. Notes 19, 9 pp. 1937. [Mimeographed.]

fertilizers. The calculation of the necessary amount of fertilizing material to be applied is a matter of simple arithmetic. If, for example, a soil analyzes 30 pounds per acre of available phosphorus pentoxide and the desired content is 80 pounds, the deficiency of 50 pounds may be corrected by an addition of about 100 pounds of 45-percent treble superphosphate or 250 pounds of 20-percent superphosphate.

The suitability of different fertilizers for nursery use, the technique of their application, and the nutrient content of a variety of natural organic remains have been previously reported[5] (*14, 15, 16*).

DISCUSSION

Although the relation of the chemical composition of soil to tree growth has received considerable attention during the past 50 years, very little reliable information has been obtained. In most of the earlier experiments a number of important conditions were overlooked or misinterpreted. The extracting solutions used in chemical analysis were usually too strong, and hence the available nutrients were not determined. No regard was paid to the distribution and availability of nutrients in different soil horizons. The reactions between the nutrient salts, colloids, and other soil constituents were not considered. The studies dealt largely with a specific constituent and disregarded the influence of numerous other physical, chemical, and biological factors. The production of dry matter alone was studied, but the physiological and anatomical development of the seedlings was ignored. Particular attention was given to the production of luxuriant vegetative growth, but not necessarily to the development of vigorous seedlings resistant to diseases and unfavorable conditions of environment. As a result of this, until recently there has been little agreement among the leaders in silviculture on the problems of nursery-soil fertility and the use of fertilizers in general, or even on the role of single factors in seedling nutrition. Even in Germany, the country with the oldest silvicultural practice, the importance of nursery-soil fertilization has been fully recognized only during the past few years. The following translation of a few sentences from Dengler's modern and generally accepted text on silviculture (*5, pp. 420–421*), shows the confusion that has existed in German nursery practice.

> The soils of forest nurseries, permanent nurseries in particular, are impoverished in mineral nutrients by forest seedlings and require regular application of fertilizers, as do agricultural soils. At least as much fertilizing material must be returned to the nursery soil as has been taken from the soil by the young plants. The earlier, often expressed idea that the planting stock for poorer sites should not be encouraged by fertilization, which has gone so far that the commercial nurseries were offering stunted seedlings as being especially well suited for reforestation of the poorer soils, is positively and absolutely false.

The opposition to the use of fertilizers in forest nurseries was largely due to the fact that the results of fertilization were often confused with the results of unskilled fertilization, particularly fertilization with unbalanced or excessive nitrogen. Thus, past experience provides but little information on the problem of metabolism and

[5] WILDE, S. A. COMMERCIAL FERTILIZERS, THE USE OF LIQUID FERTILIZERS, AND PREPARATION AND USE OF COMPOSTED FERTILIZERS IN FOREST NURSERIES. Wis. State Conserv. Dept. in coop. with Wis. Agr. Col. Tech. Notes 7, 8, and 12, 25 pp., illus. 1936. [Mimeographed.]

correct use of fertilizers. The "new deal" in seedling nutrition is in its infancy, and it may be several years before the information presented in recent literature can be clarified and erroneous conclusions discarded. Particularly is this true in regard to greenhouse and sample plot studies carried on in this country and abroad during the past few years. For this reason, it is believed that a determination of nutrients and associated growth factors in productive forest soils provides more reliable general standards for the maintenance of fertility in nursery soils than are obtained by empirical trials.

SUMMARY

The soils under productive stands of representative coniferous species (*Pinus banksiana*, *P. resinosa*, *P. strobus*, and *Picea glauca*) were analyzed for pH value, exchange capacity, total and available nitrogen, available phosphorus, available potash, and replaceable bases. By means of statistical treatment of the data obtained, standards for the maintenance of fertility in coniferous nursery soils were established. The analyses of virgin soils showed that the nitrogen-phosphoric acid-potash ratio is about 1–2–5 for all four coniferous species studied. Typological investigations in Europe and greenhouse studies at the University of Wisconsin have indicated that the standards established for *Pinus resinosa* and *Picea glauca* may be safely applied to *Pinus sylvestris* and *Picea excelsa*, respectively.

General directions for the adjustment of soil conditions are outlined.

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U. S. GOVERNMENT PRINTING OFFICE: 1939

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